PHYSIOLOGICAL TOLERANCE MECHANISMS OF SERPENTINE TOLERANT PLANTS FROM SERBIA

Dražen D. Vicić[1], Milovan M. Stoiljković[2], Nenad Č. Bojat[3],
Marko S. Sabovljević[4] & Branka M. Stevanović[4]

Résumé.— *Mécanismes de tolérance physiologique des plantes tolérantes à la serpentine en Serbie.* — Les sols de serpentine (sols ultramafiques) constituent des environnements de croissance particulièrement stressants pour les plantes en raison du déficit en macronutriments (N, P, K, Ca), de la toxicité des macronutriments (Mg ; rapport Mg/Ca extrêmement élevé), de la toxicité des micronutriments (Mn, Fe, Ni, Cu, Zn) et de la toxicité d'autres métaux lourds (Al, Cr, Co). En outre, les sols de serpentine sont souvent superficiels, rocheux et sujets à la sécheresse. En raison des conditions chimiques et physiques particulièrement défavorables, les sols de serpentine abritent une grande proportion d'espèces végétales endémiques adaptées à leur environnement austère. Nous avons analysé les concentrations d'éléments (Ca, Mg, Al, Mn, Fe, Ni, Cu et Zn) contenus dans les racines, les tiges et les tissus foliaires des taxons *Halacsya sendtneri* (Boraginaceae ; strictement endémique aux sols de serpentine), *Cheilanthes marantae* (Pteridaceae ; fortement endémique aux sols de serpentine/bon indicateur de serpentine) et *Seseli rigidum* (Apiaceae ; faible indicateur de serpentine/ indifférent au sol), poussant sur les sols serpentiniques et calcaires de Serbie. Le facteur de bioaccumulation d'éléments a été calculé en établissant le rapport entre la concentration d'éléments présents dans les tissus foliaires et la concentration d'éléments présents dans les sols et assimilables par les végétaux. Les concentrations tissulaires en Ca et Mg des taxons *H. sendtneri* et *S. rigidum* indiquent que ces espèces absorbent du Ca et le transfèrent dans les feuilles, en fonction du taux de Mg, dans le but de maintenir un rapport Mg/Ca adéquat dans les tissus. L'espèce *C. marantae* n'a présenté aucune absorption et translocation de Ca, mais a démontré une séquestration de Mg dans les racines. L'exclusion et la séquestration des métaux lourds étaient les principaux mécanismes de tolérance physiologique démontrant une tolérance à la serpentine chez les trois espèces. Le taxon *S. rigidum* a montré des divergences entre les écotypes tolérants à la serpentine et les écotypes tolérants au calcaire. Ces derniers pourront servir d'espèces modèles pour des études complémentaires sur l'adaptation physiologique des taxons aux sols présentant une composition chimique extrême.

Summary.— Serpentine (ultramafic) soils are extremely stressful environments for plant growth due to macronutrient deficiency (N, P, K, Ca), macronutrient toxicity (Mg; extremely high Mg:Ca ratio), and micronutrient toxicity (Mn, Fe, Ni, Cu, Zn), as well as toxicity of other heavy metals (Al, Cr, Co). Serpentine soils are also often shallow, rocky, and susceptible to drought. As a result of extreme adverse physical and chemical conditions, serpentine soils support a high proportion of endemic plant species that are adapted to their harsh environment. We analysed root, stem, and leaf tissue element concentrations (Ca, Mg, Al, Mn, Fe, Ni, Cu, and Zn) of *Halacsya sendtneri* (Boraginaceae; strict serpentine endemic), *Cheilanthes marantae* (Pteridaceae; broad serpentine endemic/strong indicator), and *Seseli rigidum* (Apiaceae; weak serpentine indicator/indifferent) growing on serpentine and limestone in Serbia. Element bioaccumulation factor was calculated as the ratio of plant tissue element concentration to soil plant-available element concentration. Tissue concentrations

[1] Author for correspondence: Faculty of Ecology and Environmental Protection, Union – "Nikola Tesla" University, Cara Dušana 62-64, 11000 Belgrade, Serbia. Tel: +381-64-3306468, Tel/fax: +381-11-2171-770, E-mail: vicicdra@gmail.com

[2] Department of Physical Chemistry, "Vinča" Institute of Nuclear Sciences, University of Belgrade, P. O. Box 522, 11001 Belgrade, Serbia. E-mail: missa@vinca.rs

[3] Faculty of Economics and Engineering Management, Cvećarska 2, 21000 Novi Sad, Serbia. E-mail: nbojat@yahoo.com

[4] Institute of Botany and Botanical Garden, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia. E-mail: marko@bio.bg.ac.rs ; bstev@bio.bg.ac.rs

of Ca and Mg for *H. sendtneri* and *S. rigidum* indicate that the species selectively uptake and translocate Ca to leaves, relative to Mg, to maintain adequate tissue Mg:Ca ratio. *C. marantae* did not exhibit selective Ca uptake or translocation, but did exhibit Mg sequestration in roots. Heavy metal exclusion and sequestration were the primary physiological tolerance mechanisms conveying serpentine tolerance in the three species. *S. rigidum* exhibited divergence into serpentine tolerant and limestone tolerant ecotypes, presenting a useful model species for further studies of physiological adaptation to chemically extreme soils.

Ultramafic rock (peridotite) is oceanic mantle tectonically emplaced on land along ancient continental plate margins (Brooks, 1987). Peridotite may be partially or completely hydrothermally altered to serpentinite. Peridotite and serpentinite are collectively referred to as serpentine. Due to the unique mineralogy of serpentine, serpentine soils have extremely chemically adverse conditions for plant growth including macronutrient deficiency (N, P, K, Ca), macronutrient toxicity (Mg; extremely high Mg:Ca ratio), and micronutrient toxicity (Mn, Fe, Ni, Cu, and Zn), as well as toxicity of other nonessential heavy metals (Al, Cr, Co) (Kruckeberg, 1984; Brooks, 1987; Alexander *et al.,* 2007; Kazakou *et al.,* 2008). Serpentine soils are typically very shallow, rocky, and prone to drought. The sparse vegetation on serpentine soils results in susceptibility to erosion and high fluctuation in soil temperature. This combination of physical, chemical and biotic factors that limits plant growth on serpentine is referred to as the "serpentine syndrome" (Kruckeberg, 1984; Brady *et al.,* 2005).

Several physiological adaptations have been identified that appear to be important for plant serpentine tolerance. Inherently slow growth rates and high root to shoot biomass ratios allow plants to cope with drought and low soil concentrations of plant essential macronutrients such as N, P, and K (Kruckeberg, 1984; Alexander *et al.,* 2007; O'Dell & Rajakaruna, 2011). Ca is a plant essential macronutrient required in higher concentrations than Mg (Marschner, 2002). Ca concentrations are typically very low and Mg concentration is typically extremely high in serpentine soils (Mg:Ca molar ratio >> 1.0). Ca and Mg compete for uptake at the root. The high concentration of Mg relative to Ca in serpentine soils may induce Ca deficiency in non-adapted plants. Numerous serpentine tolerant plants have been found to have Ca and Mg regulation tolerance mechanisms to cope with the high Mg:Ca mol ratio of serpentine soils including: 1) selective Ca uptake at the root and translocation to shoot, 2) Mg exclusion at the root, and 3) Mg sequestration at the root (Lyon *et al.,* 1971; Shewry & Peterson, 1975; Wallace *et al.,* 1982; Kruckeberg, 1984; O'Dell *et al.,* 2006; O'Dell & Claassen, 2006a, b; Alexander *et al.,* 2007; Turner *et al.*, 2010; O'Dell & Rajakaruna, 2011). Physiological regulation of Mg and Ca uptake allows the plant to maintain balanced tissue Mg:Ca ratios.

Heavy metal physiological tolerance mechanisms include 1) metal exclusion at the root, 2) sequestration to various plant organs, and 3) toxicity tolerance (Baker, 1987; Shaw, 1990; Alexander *et al.*, 2007; O'Dell & Rajakaruna, 2011). In some plant species, heavy metals may be selectively taken up at the root and translocated to leaves, accumulating to very high concentrations ($> 1000 \ \mu g \ g^{-1}$) in a process called "hyperaccumulation" (Brooks, 1998; O'Dell & Rajakaruna, 2011; Van der Ent *et al.,* 2013). The knowledge of plant physiological adaptations to serpentine, especially hyperaccumulation, is of particular interest in plant species selection for ecosystem restoration and phytoremediation (Salt *et al.,* 1998; Díez Lázaro *et al.,* 2006; Barzanti *et al.,* 2011). Serpentine endemic plants are a useful model for ecologists to study evolution and adaptation to extreme edaphic environments (Harrison & Rajakaruna, 2011).

Extensive areas of serpentine are located on the Balkan Peninsula. These serpentine habitats are important centers of endemism, both as active areas of speciation of neoendemics and as refugia for paleoendemics. The obligate serpentine endemic flora of the Balkan Peninsula includes taxa from 30 families and 73 genera (Stevanović *et al.,* 2003), suggesting potential for a variety of adaptation strategies to serpentine soils (Anacker, 2011; Cecchi & Selvi, 2009). Plants found on serpentine are commonly classified into one of three groups based upon their affinity to serpentine: strict endemic (exclusively on serpentine), indicator (more often on serpentine than not), and indifferent (bodenvag; equally common on or off serpentine) (Reeves *et al.*, 1999; Kazakou *et al.,* 2010; Kay *et al.,* 2011).

Little is known about the serpentine tolerance mechanisms of serpentine endemic plants from the Balkan region. The aim of this study was to identify physiological tolerance mechanisms of three serpentine tolerant plant species from Serbia including *Halacsya sendtneri* (Boraginaceae; strict endemic), *Cheilanthes marantae* (Pteridaceae; strong indicator), and *Seseli rigidum* (Apiaceae; weak indicator/indifferent). Root, stem, and leaf tissue samples along with a soil sample from the rooting zone were collected from field plants. The samples were analysed for concentrations of selected elements, and plant tissue element bioaccumulation factors were calculated to elucidate physiological tolerance mechanisms of the species to the extreme chemistry of the substrate.

MATERIALS AND METHODS

STUDY SPECIES

Halacsya sendtneri (Boiss.) Dörfl. (Boraginaceae) is a low-growing, perennial herb that occurs exclusively on rocky serpentine soils (strict endemic). The range of *H. sendtneri* extends from northern Greece to western Bosnia (Cincović & Kojić, 1974; Stevanović, B. *et al.,* 1995 ; Stevanović, V. *et al.*, 2003). *Halacsya* is a monotypic genus. The geographical isolation and relation of *H. sendtneri* to other members of tribe Lithospermae and family Boraginaceae is of substantial study interest to plant ecologists and taxonomists (Thomas *et al.,* 2008; Cecchi & Selvi, 2009; Cecchi *et al.,* 2011). Biochemists have extensively studied biological compounds in the species (Nićiforović *et al*., 2010; Mašković *et al*., 2012a, b). *Cheilanthes marantae* (L.) Domin. (Pteridaceae) is a small fern that often grows in crevices of serpentine rock outcrops (strong indicator). The range of *C. marantae* extends from Portugal in the west to the Himalayas in the east (Vukičević, 1970; Selvi, 2007; García-Barriuso *et al.,* 2011). *Seseli rigidum* Waldst. et Kit. (Apiaceae) is a perennial herb that grows on very rocky soils of diverse lithology, including limestone and serpentine (weak indicator/indifferent). The range of *S. rigidum* includes the Balkans and Ukraine (Nikolić, 1973; Dudić *et al.,* 2007).

COLLECTION LOCATIONS

Collection locations included Brđani Gorge (BR), Ravnik (RA), Đetinja River Gorge (DJ), and Ovčar Banja (OB) in Serbia (Tab. I, Fig. 1). BR, RA, and DJ are underlain by serpentine. OB is underlain by limestone.

Serpentine soil of the BR site is shallow (< 10 cm) and very rocky. Large boulders and rock outcrops are distributed across the slope. Dominant vegetation of the site is grassland. *H. sendtneri* and *S. rigidum* grow around the boulders and rock outcrops. *C. marantae* grows in crevices within the rock outcrops. Serpentine soil of the RA site is shallow with abundant organic debris from *Pinus sylvestris* trees that form the forest vegetation. Clumps of *H. sendtneri* grow on the forest floor. Serpentine substrate of the DJ site is primarily talus. *C. marantae* grows in dense, but dispersed clumps on the talus. The OB site limestone habitat is bounded by a highway on one side and vertical cliff on the other. *S. rigidum* grows scattered at the bottom of the cliff.

TABLE I

Collection location substrate, location coordinates, altitude, aspect, and slope

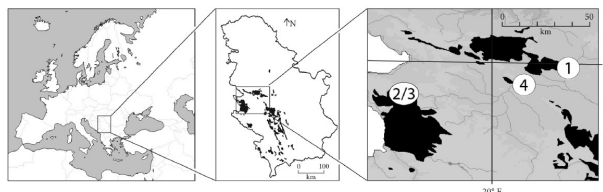| Nr.	Collection location	Substrate	Location coordinates		Altitude	Aspect	
			Latitude	Longitude	meters	direction	slope °
1	Brđani Gorge (BR)	serpentine	43°59'22.1"	20°25'34.2"	383	SW	45
2	Ravnik (RA)	serpentine	43°51'39.5"	19°35'4.7"	616	S-SW	30
3	Đetinja River Gorge (DJ)	serpentine	43°51'22.9"	19°35'40.1"	600	E-SE	65
4	Ovčar Banja (OB)	limestone	43°54'0.8"	20°11'47.6"	294	NW	70

Figure 1.— Serbia (L); Distribution of serpentine (black) in Serbia (M); Collection locations 1 to 4 (R) (see Table I for details).

Plant tissue collection and analysis

Samples were collected in 2011. Roots, stems and leaves were sampled from five different individuals of *H. sendtneri* growing on serpentine at both BR and RA. Rhizomes and fronds were sampled from five different individuals of *C. marantae* growing on serpentine at both BR and DJ. The fronds of *C. marantae* were separated into stipes and leaves. Roots, stems, and leaves were sampled from five different individuals of *S. rigidum* growing on serpentine at BR and growing on limestone at OB. There was at least 10 meters distance between each individual plant collected.

All plant samples were thoroughly washed with distilled water, air dried, and ground to a fine powder. Samples of approximately 0.5 g were weighed and individually digested in glass beakers with a 2:1 mixture of HNO_3 (65 % p.a. Carlo Erba Reagents, Italy) and H_2O_2 (30 % p.a. VWR International, USA). Plant tissue digests were then diluted with double distilled water to 50 ml. Plant digests were analysed for Ca, Mg, Al, Mn, Fe, Ni, Cu, and Zn with Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Spectroflame, Germany).

Soil sampling and analysis

For three of the five plant tissue sample collections per species, a soil sample was collected from within the rooting zone of the plant (10 cm depth; 2 L. of soil). Soil samples were air dried and sieved to < 2 mm. Soil pH was determined in 1:5 (v/v) suspension of soil in 1 M KCl with a glass electrode (ISO 10390:1994). Bioavailable metals are extracted from 1 g of oven-dried (105 °C) soil, through sequential extraction of Zeien and Brümmer (1989) (Kaupenjohann & Wilcke, 1995; Al-Najar *et al.,* 2003; Ratuzny *et al.,* 2009; Grawunder *et al.,* 2009). This method extracts metals bound to soil with different strengths. The first four fractions are regarded as bioavailable. The first fraction represents free metals in soil solution (extracted with 1 M NH_4NO_3), the second fraction includes extracted metals that were adsorbed to the cation exchange complex (extracted with 1 M CH_3COONH_4 and 1 M NH_4NO_3), the third fraction includes extracted metals that were bound to Mn-oxides (extracted with 0.1 M $NH_2OH \cdot HCl$ + 1 M CH_3COONH_4; pH 6), and the fourth fraction includes metals that were bound to organic matter (extracted with 0.025 M NH_4-EDTA; pH 4.6). Extracts were analysed with an ICP-OES for Ca, Mg, Al, Mn, Fe, Ni, Cu, and Zn.

Data analysis

Leaf element bioaccumulation factors (BF) were calculated for all three plant species as leaf elemental concentration divided by the mean plant-available soil element concentration (Baker *et al.,* 1994). Significance of difference was tested with One-way ANOVA and individual levels of significance are indicated in the text.

RESULTS

The pH value was mildly alkaline in limestone soil and slightly acidic in serpentine soils (Tab. II). Serpentine soils contained significantly higher bioavailable Mg and Ni than the limestone soil (F = 11.064, 15.44, respectively; $p < 0.01$). The limestone soil contained significantly higher plant-available Ca, Cu, and Zn than the serpentine soils (F = 10.120, 70.117, 16.726, respectively; $p < 0.01$). There was no significant difference between the limestone and serpentine soils for Al, Mn, and Fe ($p > 0.05$). Plant-available molar Mg:Ca molar ratio was between 0.02 and 0.15 for limestone soil and ranged from 1.2 to 6.2 for the serpentine soils. Ca, Al, Mn, Fe, Ni, and Cu did not vary significantly among serpentine soils ($p > 0.05$). Zn and Mg from DJ site was significantly lower than in RA (F = 5.457, 5.85, respectively; $p < 0.05$).

Fig. 2 and Tab. III present plant tissue element concentrations and Mg:Ca molar ratios and Fig. 3 presents element bioaccumulation factors. *H. sendtneri* had significantly lower root, stem, and leaf Mg:Ca molar ratio than the soil (BR: F = 52.759, RA: F = 32.115, $p < 0.001$) indicating strong, selective Ca uptake and translocation to all plant organs. *C. marantae* had significantly higher rhizome than leaf Mg concentration (BR: F = 88.515, DJ: F = 89.177, $p < 0.001$) indicating moderate Mg sequestration to rhizomes; however the species did not maintain a favourable leaf Mg:Ca molar ratio. *S. rigidum* had significantly lower leaf Mg:Ca molar ratio than the serpentine soil (F = 88.171, $p < 0.001$) indicating strong, selective Ca uptake and translocation to leaves when grown on the very high Mg:Ca molar ratio serpentine soil. *S. rigidum* had significantly higher leaf Mg:Ca molar ratio than the limestone soil (F = 59.151, $p < 0.001$) indicating strong, selective Mg uptake and translocation to leaves when grown on the very low Mg:Ca molar ratio limestone soil. Based on bioaccumulation factors, in general, all species exhibited exclusion of Mn, Fe, and Ni at the root level. There was little regulation of the uptake and translocation of Al (BF~1). Most Al was sequestered in roots of *C. marantae* (BR: F = 17.581; DJ: F = 8.456, $p < 0.01$) and *S. rigidum* (BR: F = 31.822, OB: F = 6.89, $p < 0.05$). In general, there was uptake and translocation to leaves of Cu and Zn with the translocation of Cu and Zn to leaves in *C. marantae* being quite elevated.

TABLE II

Soil pH, bioavailable element concentration (mg kg⁻¹), and Mg:Ca molar ratio of the collection sites. Mean ± SE; n = 3

	pH	Ca	Mg	Mg:Ca	Al	Mn	Fe	Ni	Cu	Zn
BR	6.2±0.23	1667±463	2869±603	3.0±0.3	75±7	867±171	570±30	217±74	3.9±0.2	4.6±1.6
RA	6.2±0.33	4232±1733	4693±630	2.4±0.7	53±13	751±149	712±174	207±17	3.7±0.5	10.6±3.0
DJ	6.5±0.15	730±49	2266±241	5.1±0.5	33±7	781±161	563±34	175±23	2.5±0.2	1.6±0.5
OB	7.1±0.22	43215±24831	594±153	0.1±0.04	61±8	488±118	461±137	32±7	16.2±2.8	59.3±24.8

TABLE III

Root, stem, and leaf elemental concentration (mg kg⁻¹) of H. sendtneri, C. marantae, and S. rigidum. Mean ± SE; n=5. nd – not detected

| sp	site	organ	Ca	Mg	Mg:Ca	Al	Mn	Fe	Ni	Cu	Zn
H. sendtneri	BR	root	10587±977	2974±200	0.50±0.04	20.7±5.9	27.7±3.8	108±14	7±2.6	5.6±1.4	26.8±2.0
		stem	8210±342	2629±386	0.53±0.08	37.2±10	15.3±3.9	69±8.0	3.8±1.8	nd	14.1±2.6
		leaf	12204±885	3771±467	0.58±0.10	66.6±8.1	32.2±2.1	78±7.0	5±1.3	3.9±1.2	17.4±4.4
	RA	root	10758±657	3778±158	0.59±0.02	26.7±7.2	27.9±4.0	89±18	3.6±1.9	7.2±1.4	36±3.0
		stem	7892±332	2584±103	0.55±0.03	21.6±7.1	7.2±3.0	108±33	6±1.7	4.1±1.6	15.9±1.4
		leaf	23339±1099	4846±330	0.34±0.01	57.9±9.7	45.8±5.6	83±12	13.4±1.3	5.2±1.4	26.6±4.6
C. marantae	BR	rhizome	3946±457	6006±530	2.79±0.43	129±18.7	30.3±6.1	942±186	72.8±13	1.4±1.0	28.1±1.6
		stipe	2093±268	883±58	0.75±0.07	45.2±12.8	5.1±0.4	32.5±1.9	15.1±3.9	2.9±1.2	41.9±3.5
		leaf	2018±97	1503±78	1.23±0.05	33.4±3.9	15.8±2.1	102±18	15.8±1.5	9±1.0	42.5±2.8
	DJ	rhizome	3106±324	5437±319	2.99±0.12	140±28.3	32.8±4.0	1142±139	69.2±3.7	nd	17.7±2.5
		stipe	1789±454	1026±141	1.77±0.58	54.7±11.4	15.7±4.6	74.4±12	10.8±5.3	4.8±2.1	37±8.9
		leaf	1201±104	1987±75	2.89±0.28	31.2±5.2	20.3±3.1	153±19	20.7±3.3	6.8±1.7	36.3±4.8
S. rigidum	BR	root	4111±148	3410±253	1.37±0.09	87.7±16.4	44±5.4	410±67	14.3±2.6	1.9±1	50.4±3.3
		stem	2477±61	2393±125	1.62±0.13	nd	14.6±1.8	19±2.0	4.9±1.6	2.9±0.9	22.8±2.5
		leaf	9521±528	3689±605	0.66±0.12	20.5±5.3	86.2±8.9	73±8.0	0.9±0.9	0.7±0.7	46.3±5.0
	OB	root	7226±582	2613±272	0.63±0.09	153±45	38.8±3.6	218±45	1.6±1.3	13.3±2.1	73.8±6.8
		stem	4287±296	1428±139	0.56±0.05	7.3±3.7	15.2±1.7	45±5.0	0.7±0.4	5.7±1.2	37.8±1.6
		leaf	13615±657	2605±167	0.32±0.01	53.3±20.1	77.6±8.9	97±13	2.7±1.2	1.5±0.5	60.1±8.0
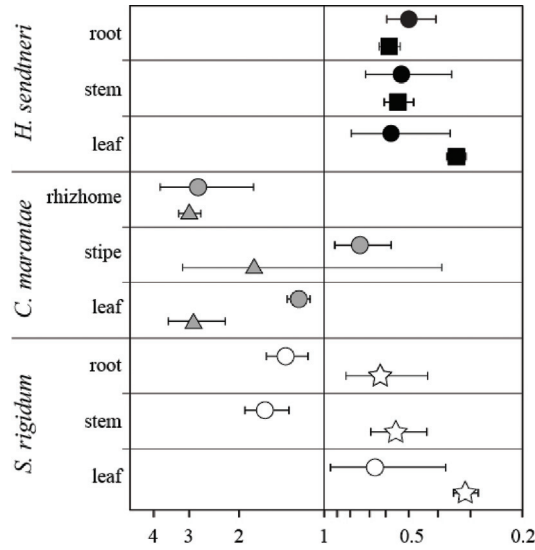
Figure 2.— Root, stem, and leaf Mg:Ca molar ratio (Mean, 95 % confidence intervals; n=5) of *H. sendtneri* (black), *C. marantae* (gray), and *S. rigidum* (white); Circle – site BR (serpentine), square – site RA (serpentine), triangle – site DJ (serpentine), star – site OB (limestone); Plant tissue Mg:Ca molar ratio > 1.0 is unfavourable; Mg:Ca molar ratio < 1.0 is favourable.
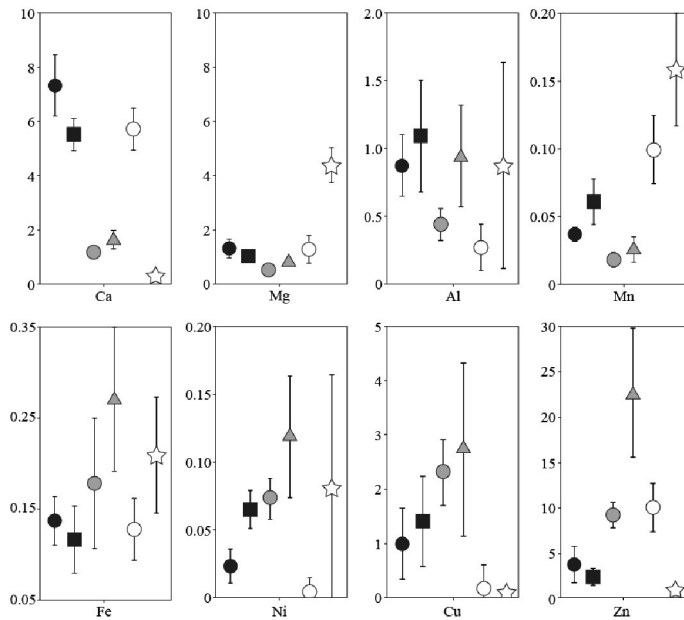

Figure 3.— Element bioaccumulation factor in leaves. Mean, 95 % confidence interval; n=5. *H. sendtneri* (black), *C. marantae* (gray), *S. rigidum* (white); Circle – site BR (serpentine), square – site RA (serpentine), triangle – site DJ (serpentine), star – site OB (limestone).

DISCUSSION

Regulation of Mg and Ca uptake at the roots and translocation to stems and leaves is a frequently documented physiological adaptation to serpentine (Lyon *et al.,* 1971; Shewry & Peterson, 1975; Wallace *et al.,* 1982; Kruckeberg, 1984; O'Dell *et al.,* 2006; O'Dell & Claassen, 2006a, b; Alexander *et al.,* 2007; Turner *et al.*, 2010; O'Dell & Rajakaruna, 2011). Ca and Mg regulation tolerance mechanisms to cope with the high Mg:Ca molar ratio of serpentine soils that have been identified include: 1) selective Ca uptake at the root and translocation to shoot, 2) Mg exclusion at the root, and 3) Mg sequestration at the root (Lyon *et al.,* 1971; Shewry & Peterson, 1975; Wallace *et al.,* 1982; Kruckeberg, 1984; O'Dell *et al.,* 2006; Alexander *et al.,* 2007; Turner *et al.*, 2010; O'Dell & Rajakaruna, 2011). Physiological regulation of Ca and Mg allows the plant to maintain balanced tissue Mg:Ca molar ratios and adequate supply of both cations.

Ca is a phloem immobile, plant-essential macronutrient (Marschner, 2002). As a result, the Ca demand of developing plant tissues in the shoot must be supplied by the xylem following uptake at the root. Root apical meristems acquire Ca directly from the surrounding soil (Marschner & Richter, 1974). Ca^{2+} and Mg^{2+} compete for uptake at the root. Excess of Mg relative to Ca can induce Ca deficiency when the plant has poor ability to selectively uptake Ca over Mg. Ca deficiency is manifested in shoots as deformity and necrosis of developing tissues including shoot apical meristem, young leaves, and fruits. Root apical necrosis is often the first manifestation of Ca deficiency in serpentine intolerant plants as roots cease to elongate and root tips become necrotic (O'Dell & Claassen, 2006a, b).

The primary role of Ca in plant tissue structural integrity is in pectin contained in cell walls (Marschner, 2002). The Type I cell walls found in dicotyledonous plants are pectin-rich. Pectin is a major component of the middle lamella, where it serves to bind cells together. Pectin is dependent upon Ca bridging for binding strength. When Ca supply to developing plant tissue is insufficient, pectin loses binding strength, cells separate, and tissues become deformed and susceptible to necrosis. The Type II cell wall of commelinoid monocotyledonous plants (Poaceae, Cypercaeae, Juncaceae, Arecaceae) and Type III cell wall of ferns and other Pteridophytes (Silva *et al.*, 2011) contain a much lower proportion of pectins and therefore, plant species in these groups have a lower Ca requirement (more tolerant of low soil Ca availability) to maintain cell wall integrity (Jarvis *et al.*, 1988; Marschner, 2002; Broadley *et al.*, 2003; Vogel, 2008; Silva *et al.*, 2011). The much lower Ca demand of plant species with Type II and Type III cell walls could convey preadaptation to the low Ca availability of serpentine soils.

In this study, *H. sendtneri* (Type I cell wall) exhibited strong, selective Ca uptake at the roots and translocation to stems and leaves as an evolved adaptation to serpentine soils. This physiological tolerance mechanism allowed *H. sendtneri* to maintain adequate tissue Mg:Ca molar ratios < 0.6 (Ca bioaccumulation factor > 5) while the serpentine soil Mg:Ca molar ratio was only 1.2 – 3.6. Selective Ca uptake at the roots and translocation to the shoot has also been documented in serpentine tolerant populations of *Phacelia californica* (Boraginaceae; weak indicator/indifferent; Type I cell wall) in California, USA (Kruckeberg, 1950). *Phacelia californica* has evolved serpentine tolerant ecotypes with selective Ca uptake and translocation to the shoot in response to the low Ca availability of serpentine soils.

S. rigidum (Type I cell wall) from BR exhibited a similar trait of strongly elevated Ca uptake at the roots and translocation to leaves (bioaccumulation factor > 5) when grown on serpentine (soil Mg:Ca molar ratio 2.6 – 3.5) which has a deficiency of Ca relative to Mg. Interestingly, in contrast, *S. rigidum* from OB exhibited strongly elevated Mg uptake and translocation to leaves (bioaccumulation factor > 4) when grown on limestone (soil Mg:Ca molar ratio 0.02 – 0.15) which has a deficiency of Mg relative to Ca. On both substrate types, *S. rigidum* was able to maintain a leaf Mg:Ca molar ratio between 0.3 and 0.7, even though the soil Mg:Ca molar ratio was as high as 3.5 on serpentine and as low as 0.02 on limestone. Similar results for

serpentine and limestone populations of *S. rigidum* were obtained by Dudić *et al*. (2007). The differing physiological response of the two *S. rigidum* populations suggests ecotypic divergence into distinct serpentine tolerant and limestone tolerant ecotypes.

C. marantae exhibited Mg sequestration to rhizomes, but did not maintain a favourable leaf Mg:Ca ratio (1.23 – 2.89). *Pteridium aquilinum* (Pteridaceae) grown on serpentine in Poland was also documented to not maintain a favourable leaf Mg:Ca ratio (1.36; Samecka-Cymerman *et al*., 2009). Ferns contain low pectin Type III cell walls which may convey preadaptation to the low Ca availability of serpentine soils.

Heavy metal physiological tolerance mechanisms previously identified in serpentine tolerant plants include 1) metal exclusion at the root, 2) sequestration to various plant organs, and 3) toxicity tolerance (Baker, 1987; Shaw, 1990; Alexander *et al.*, 2007; O'Dell & Rajakaruna, 2011). In general, all three species appeared to be excluders of Mn, Fe, and Ni. Although there was little regulation in the uptake and translocation of Al, the Al was generally sequestered in roots and rhizomes. Sequestration of heavy metals in roots is a general, common heavy metal tolerance mechanism (Baker, 1987; Shaw, 1990). In general, there was elevated uptake and translocation to leaves of Cu and Zn for all three species with the translocation of Cu and Zn to leaves in *C. marantae* being quite elevated. The leaf Cu and Zn concentrations were too low to qualify the species as a hyperaccumulator or to convey any other apparent evolutionary advantage.

CONCLUSION

Just as the serpentine syndrome is a combination of physical and chemical stresses, plant serpentine tolerance is a combination of multiple morphological and physiological adaptations. As such, it can be difficult to identify what adaptations are primarily responsible for a plant's tolerance to the environmental stresses of its habitat. This study focused on the physiological adaptations of only three species involving a limited number of elements. Even with its limited scope, the study found evidence of Ca, Mg, and heavy metal uptake and translocation regulation as serpentine tolerance adaptations. Selective Ca uptake and translocation to the leaves appears to be an important serpentine tolerance adaptation in the serpentine endemic *H. sendtneri* and the serpentine tolerant ecotype of *S. rigidum*. Mg sequestration to roots was present in *C. marantae*, but the species may benefit more from its low pectin Type III cell walls and the inherent tolerance of low soil Ca availability that it conveys. Heavy metal tolerance mechanisms including exclusion, sequestration, and tolerance appeared to be present in all three species.

Physiological adaptations to extreme substrates provide a glimpse into the evolution of a species into edaphic ecotypes and under conducive conditions, speciation into edaphic endemics (Harrison & Rajakaruna, 2011). *H. sendtneri* and *Paramoltkia doerfleri* are monotypic and both are strict serpentine endemics (Cecchi & Selvi, 2009). A genetic study of the two species and closely related taxa revealed that *H. sendtneri* and *P. doerfleri* evolved from the same nonserpentine ancestor. Additionally, serpentine tolerance has arisen at least three other times in Lithospermae (Cecchi & Selvi, 2009). The identification of *S. rigidum* serpentine tolerant (selective Ca uptake) and limestone tolerant (selective Mg uptake) ecotypes provides yet another model species with which to study physiological adaptation to extreme soils and the evolution of edaphic endemism.

REFERENCES

ALEXANDER, E.B., COLEMAN, R.G., KEELER-WOLF, T. & HARRISON, S. (2007).— *Serpentine geoecology of Western North America: Geology, soils, and vegetation*, Oxford University Press, New York, USA.

AL-NAJAR, H., SCHULZ, R. & RÖMHELD, V. (2003).— Plant availability of thallium in the rhizosphere of hyperaccumulator plants: a key factor for assessment of phytoextraction. *Plant Soil,* 249: 97-105.

ANAKER, B. (2011).— Phylogenetic patterns of endemism and diversity. Pp 49-70 *in:* S.P. Harrison & N. Rajakaruna (eds) *Serpentine: Evolution and ecology of a model system*. University of California Press, Berkeley, California, USA.

BAKER, A.J.M. (1987).— Metal tolerance. *New Phytol*., 106: 93-111.

BAKER, A.J.M., REEVES, R.D. & HAJAR, A.S.M. (1994).— Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). *New Phytol.,* 127: 61-68.

BARZANTI, R., COLZI, I., ARNETOLI, M., GALLO, A., PIGNATTELLI, S., GABBRIELLI, R. & GONNELLI, C. (2011).— Cadmium phytoextraction potential of different *Alyssum* species. *J. Hazard. Mater.,* 196: 66-72.

BROADLEY, M.A., BOWEN, H.C., COTTERILL, H.L., HAMMOND, J.P., MEACHAM, M.C., MEAD, A. & WHITE, P.J. (2003).— Variation in the shoot calcium content of angiosperms. *J. Exp. Bot.,* 54: 1431-1446.

BRADY, K.U., KRUCKEBERG, A.R. & BRADSHAW JR, H.D. (2005).— Evolutionary ecology of plant adaptation to serpentine soils. *Annu. Rev. Ecol. Evol. Syst.,* 36: 243-266.

BROOKS, R.R. (1987).— *Serpentine and its vegetation: A multidisciplinary approach*. Dioscorides Press, Portland, OR, USA.

BROOKS, R.R. (1998).— *Plants that hyperaccumulate heavy metals*. CAB International, Wallingford, UK.

CECCHI, L., COPPI, A. & SELVI, F. (2011).— Evolutionary dynamics of serpentine adaptation in *Onosma* (Boraginaceae) as revealed by ITS sequence data. *Plant. Syst. Evol.,* 297: 185-199.

CECCHI, L. & SELVI, F. (2009).— Phylogenetic relationships of the monotypic genera *Halacsya* and *Paramoltkia* and the origins of serpentine adaptation in circum-mediterranean Lithospermeae (Boraginaceae): insights from ITS and *mat*K DNA sequences. *Taxon,* 58: 700-714.

CINCOVIĆ, T. & KOJIĆ, M. (1974).— Fam Boraginaceae A. L. Jussieu 1789. Pp 52-53 *in*: M. Josifović (ed). *Flora SR Srbije 6*. Serbian Academy of Sciences and Arts, Belgrade, Serbia.

DÍEZ LÁZARO, J., KIDD, P.S. & MONTERROSO MARTÍNEZ, C.A. (2006).— Phytogeochemical study of the Trás-os-Montes region (NE Portugal): Possible species for plant-based soil remediation technologies. *Sci. Total Environ.,* 354: 265-277.

DUDIĆ, B., RAKIĆ, T., ŠINŽAR-SEKULIĆ, J., ATANACKOVIĆ, V. & STEVANOVIĆ, B. (2007).— Differences of metal concentrations and morpho-anatomical adaptations between obligate and facultative serpentinophytes from western Serbia. *Arch. Biol. Sci.,* 59: 341-349.

GARCÍA-BARRIUSO, M., BERNARDOS, S., NABAIS, C., PEREIRA, D. & AMICH, F. (2011).— Phytogeochemical, geographical and vulnerability study of the Paleosubtropical element *Notholaena marantae* subsp. *marantae* (Sinopteridaceae) at the western edge of its range. *Biologia,* 66(2): 258-265.

GRAWUNDER, A., LONSCHINSKI, M., MERTEN, D. & BÜCHEL, G. (2009).— Distribution and bonding of residual contamination in glacial sediments at the former uranium mining leaching heap of Gessen/Thuringia, Germany. *Chem. Erde,* 69 (S2): 5-19.

HARRISON, S.P. & RAJAKARUNA, N. (2011).— What have we learned from serpentine about evolution, ecology, and other sciences. Pp 417-427 *in:* Harrison, S.P. & Rajakaruna, N. (eds). *Serpentine: Evolution and ecology of a model system*. University of California Press, Berkeley, California, USA.

ISO 10390 (1994).— *Soil quality - Determination of pH*. International Organization for Standardization, Geneva, Switzerland.

JARVIS, M.C., FORSYTH, W. & DUNCAN, H.J. (1988).— A survey of the pectic content of non-lignified monocot cell walls. *Plant Physiol.,* 88: 309-314.

KAUPENJOHANN, M. & WILCKE, W. (1995).— Heavy metal release from a serpentine soil using a pH-stat technique. *Soil Sci. Soc. Am. J.,* 59: 1027-1031.

KAY, K.M., WARD, K.L., WATT, L.R. & SCHEMSKE, D.W. (2011).— Plant speciation. Pp 71-96 *in:* S.P. Harrison & N. Rajakaruna (eds) *Serpentine: Evolution and ecology of a model system*. University of California Press, Berkeley, California, USA.

KAZAKOU, E., ADAMIDIS, G.C., BAKER, A.J.M., REEVES, R.D., GODINO, M. & DIMITRAKOPOULOS, P.G. (2010).— Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. *Plant Soil,* 332: 369-385.

KAZAKOU, E., DIMITRAKOPOULOS, P.G., BAKER, A.J.M., REEVES, R.D. & TROUMBIS, A.Y. (2008).— Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: From species to ecosystem level. *Biol. Rev.,* 83: 495-508.

Kruckeberg, A.R. (1950).— *An experimental inquiry into the nature of endemism on serpentine soils.* Ph.D. dissertation. University of California, Berkeley.

Kruckeberg, A.R. (1984).— *California serpentines: flora, vegetation, geology, soils, and management problems.* University of California Press, Berkeley, USA.

Lyon, G.L., Peterson, P.J., Brooks, R.R. & Butler, G.W. (1971).— Calcium, magnesium and trace elements in a New Zealand serpentine flora. *J. Ecol.,* 59: 421-429.

Marschner, H. (2002).— *Mineral nutrition of higher plants.* Academic Press, San Diego, California, USA.

Marschner, H. & Richter, C. (1974).— Calcium-transport in Wurzeln von Mais- und Bohnenkeimpflanzen. *Plant Soil*, 40: 193-210.

Mašković, P., Maksimović, J.D., Maksimović, V., Blagojević, J., Vujošević, M., Manojlović, N.T., Radojković, M., Cvijović, M. & Solujić, S. (2012a).— Biological activities of phenolic compounds and ethanolic extract of *Halacsya sendtneri* (Boiss) Dörfler. *Cent. Eur. J. Biol.,* 7: 327-333.

Mašković, P.Z., Manojlović, N.T., Mandić, A.I., Mišan, A.C., Ivan, L., Radojković, M.M., Cvijović, M.S. & Solujić, S.R. (2012b).— Phytochemical screening and biological activity of extracts of plant species *Halacsya sendtneri* (Boiss.) Dörfl, *Hemijska Industrija*, 66: 43-51.

Nićiforović, N., Mihailović, V., Mašković, P., Solujić, S., Stojković, A. & Muratspahić, D.P. (2010). — Antioxidant activity of selected plant species; potential new sources of natural antioxidants. *Food Chem. Toxicol.,* 48: 3125-3130.

Nikolić, V. (1973).— *Seseli rigidum* Waldst. et Kit. P. 242 *in*: M. Josifović (ed.). *Flora SR Srbije 6*. Serbian Academy of Sciences and Arts, Belgrade, Serbia.

O'Dell, R.E. & Claassen, V.P. (2006a).— Serpentine and nonserpentine *Achillea millefolium* accessions differ in serpentine substrate tolerance and response to organic and inorganic amendments. *Plant Soil*, 279: 253-269.

O'Dell, R.E. & Claassen, V.P. (2006b).— Vertical distribution of organic amendment influences the rooting depth of revegetation species on barren, subgrade serpentine substrate. *Plant Soil*, 285: 19-29.

O'Dell, R.E., James, J.J. & Richards, J.H. (2006).— Congeneric serpentine and nonserpentine shrubs differ more in leaf Ca:Mg than in tolerance of low N, low P, or heavy metals. *Plant Soil*, 280: 49-64.

O'Dell, R.E. & Rajakaruna, N. (2011).— Intraspecific variation, adaptation, and evolution. Pp 97-137 *in:* S.P. Harrison & N. Rajakaruna (eds). *Serpentine: Evolution and ecology of a model system*. University of California Press, Berkeley, California, USA.

Ratuzny, T., Gong, Z. & Wilke, B.-M. (2009).— Total concentrations and speciation of heavy metals in soils of the Shenyang Zhangshi Irrigation Area, China. *Environ. Monit. Assess.,* 156: 171-180.

Reeves, R.D., Baker, A.J.M., Borhidi, A. & Berazaín, R. (1999).— Nickel hyperaccumulation in the serpentine flora of Cuba. *Ann.Bot.,* 83: 29-38.

Salt, D.E., Smith, R.D. & Raskin, I. (1998).— Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 49: 643-668.

Samecka-Cymerman, A., Garbiec, K., Kolon, K. & Kempers, A.J. (2009).— Factor analysis of the elemental composition of *Pteridium aquilinum* from serpentine and granite soils as a tool in the classification of relations between this composition and the type of parent rock in the Ślęża Massif in Lower Silesia, Poland. *Environ. Geol.*, 58: 509-514.

Selvi, F. (2007).— Diversity, geographic variation and conservation of the serpentine flora of Tuscany (Italy). *Biodivers. Conserv.,* 16: 1423-1439.

Shaw, A.J. (1990).— *Heavy metal tolerance in plants: Evolutionary aspects*. CRC Press, Boca Raton, Florida, USA.

Shewry, P.R. & Peterson, P.J. (1975).— Calcium and magnesium in plants and soil from a Shetland area on Unst, Shetland. *J. Appl. Ecol.,* 12: 381-391.

Silva, G.B., Ionashiro, M., Carrara, T.B., Crivellari, A.C., Tine, M.A.S., Prado, J., Carpita, N.C. & Buckeridge, M.S. (2011).— Cell wall polysaccharides from fern leaves: Evidence for a mannan-rich Type III cell wall in *Adiantum raddianum*. *Phytochemistry,* 72: 2352-2360.

Stevanović, B., Glišić, O. & Šinžar, J. (1995).— Ecology, distribution and protection of endemo-relict serpentinophyte *Halacsya sendtneri* (Boiss.) Dorfl. *7th European Ecological Congress, EURFCO,* Budapest, p. 67.

Stevanović, V., Tan, K. & Iatrou, G. (2003).— Distribution of the endemic Balkan flora on serpentine I. – Obligate serpentine endemics. *Plant Syst. Evol.,* 242: 149-170.

Thomas, D.C., Weigend, M. & Hilger, H.H. (2008).— Phylogeny and systematics of *Lithodora* (Boraginaceae-Lithospermeae) and its affinities to the monotypic genera *Mairetis*, *Halacsya* and *Paramoltkia* based on ITS1 and $trnL_{UAA}$-sequence data and morphology. *Taxon,* 57: 79-97.

Turner, T.L., Bourne, E.C., Von Wttberg, E.J., Hu, T.T. & Nuzhdin, S.V. (2010).— Population resequencing reveals local adaptation of *Arabidopsis lyrata* to serpentine soils. *Nat. Genet.*, 42: 260-263.

Van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J. & Schat, H. (2013).— Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant Soil,* 362: 319-334.

Vogel, J. (2008).— Unique aspects of the grass cell wall. *Curr. Opin. Plant Biol.,* 11: 301-307.

Vukičević, E. (1970).— *Cheilanthes marantae* (L.) Domin. P. 81 *in*: M. Josifović (ed.). *Flora SR Srbije 1.* Serbian Academy of Sciences and Arts, Belgrade, Serbia.

Wallace, A., Jones, M.B. & Alexander, G.V. (1982).— Mineral composition of native woody plants growing on a serpentine soil in California. *Soil Sci*., 134: 42-44.

Zeien, H. & Brümmer, G.W. (1989).— Chemische Extraktion zur Bestimmung von Schwermetallbindungsformen in Böden. *Mitt. Dtsch. Bodenkdl. Ges.,* 59: 505-515.