INTRODUCTION

Serpentine rocks and soils are ubiquitous on the Balkan Peninsula, but always patchily distributed. On the territory of Serbia, there are both broad and narrow serpentine zones distinctly delimited from the adjacent surrounding regions with a different geological substratum (e.g., limestone). Serpentine areas in Western (the Brđanska Gorge, Mts. Suvobor, Maljen, Tara, Zlatibor, Mokra Gora, etc.), Central (Mts. Goč, Stolovi, and Kopaonik, the Raška region, the Ibar River valley), and Southwest (the surroundings of Peć, Orahovac, and Koznik) Serbia are more frequently of Jurassic and seldom of Paleozoic age.

As is well known, serpentine soils contain disproportionately large quantities of mafic minerals (magnesium and iron compounds) and some trace elements (Ni, Cr, Co), whereas the quantity of calcium is low, as is that of some other elements essential to plants, such as nitrogen, potassium, and phosphorus (Kruckeberg, 1954; Proctor and Woodel, 1975; Brady et al., 2005). The unfavorable mineral composition of the shallow, alkaline soil and other adverse environmental conditions (water shortage, high temperatures, intense insolation) result in scarce depauperate vegetation. Serpentine landscapes are usually referred to as barren. On serpentine outcrops, frequently stunted herbaceous and shrubby plants are dominant. Due to specific structural and functional adaptive features, such plants are classified in the ecological group of serpentinophytes (Medina et al., 1994; O’Dell et al., 2006; Proctor, 1970; Kruckeberg, 1984; Specht et al., 2001).

Most often, serpentinophytes are characterized by nanism, a sparsely-branched growth habit, stenophylism, an atypical (sparce) indumentum, and (sometimes) a glaucous and glabrous leaf surface and stout root system (Pichi-Sermolli, 1948; Brady et al., 2005). In general, deficiency of water and indispensable mineral elements results in reduction of the above-ground plant parts and a high root to stem ratio. Numerous structural and functional adaptations of the plants from serpentinophytes are referred to as the "serpentine syndrome", a term coined by Jenny (1980). Adaptive traits that evolved along the evolutionary trajectory have rendered serpentinophytes capable not only of tolerating manifold stressful conditions, but also of competing successfully with other plants in such...
habitats. (Proctor, 1971; Harrison, 1999; Brady et al., 2005).

Serpentine habitats in Serbia, as worldwide, are colonized by (a) serpentine-facultative plants and (b) serpentine-obligate plants, i.e., species which grow exclusively on serpentine soils and are not to be found on other types of substrate (Stevanović et al., 2003). Serpentine-obligate plants in the Balkans are most often endemic serpentine species, and their habitats are sites of floristic differentiation and speciation in this part of Europe (Stevanović et al., 1995; Stevanović et al., 2003). Between serpentine-facultative and serpentine-obligate plants, there are certain differences of structural and functional traits enabling them to cope with nutrient and other environmental stresses. For this reason, plant edaphic specialization is of exceptional interest, particularly when endemic species are in question.

The present study is concerned with differences in serpine tolerance adaptations among two Balkan-endemic obligate serpentinoephytes, Fumana bonapartei Maire & Petitmengin and Stachys recta var. chrysophaea (Pančić) Hayek, and the facultative serpentinephyte Seseli rigidum Waldst & Kit, thriving in contrasting serpentine and calcareous habitats. The investigations encompass analysis of the quantity of magnesium, calcium, iron, nickel, and chromium in roots, stems, and leaves, as well as analysis of morpho-anatomical structure of leaves and stems in all three plant species.

MATERIALS AND METHODS

Calcium, magnesium, iron, nickel, and chromium quantities were determined in the roots, stems, and leaves of plants from serpentine of a Jurassic deposit in the region of the Tara massif in Western Serbia. Plant material of the obligate serpentinoephytes Fumana bonapartei Maire & Petitmengin and Stachys recta var. chrysophaea (Pančić) Hayek, and the facultative serpentinoiphyte Seseli rigidum Waldst & Kit, thriving in contrasting serpentine and calcareous habitats. The investigations encompass analysis of the quantity of magnesium, calcium, iron, nickel, and chromium in roots, stems, and leaves, as well as analysis of morpho-anatomical structure of leaves and stems in all three plant species.

Sample preparation and analytical procedure: mineral element analysis

Plant samples were washed in fresh water and rinsed with distilled water. Dry weights were obtained after oven drying at 60°C for two days. For each sample, 0.5 g of dry and finely crushed plant material was mixed with 10 ml of concentrated HNO₃ and incubated overnight. This solution was given 5 ml of concentrated H₂SO₄, and the sample was gradually heated until a temperature of 110-120°C was reached. When the sample started boiling and turned brown, 1-2 ml of HNO₃ was added, drop by drop, to make the sample clear. After cooling, the solution was diluted with de-ionized water to a volume of 50 ml (ISO standard 6636/2). Concentrations of Ca, Mg, Fe, Ni, and Cr in the solution were measured on an atomic absorption spectrophotometer (GBC-AVANTA) and determined relative to the solution absorbance of such elements in already known concentrations.

Anatomical analyses were done on permanent slides of leaves and stems prepared by the standard method for light microscopy (Ruzin, 1999). Plant material was fixed overnight in Navasihin's fixative, dehydrated in ethanol, and embedded in paraplast (Ruzin, 1999). Thin cross sections (5 µm thick) of leaves, stems, and roots were cut on a Reichert sliding microtome and stained with both safranine and hematoxylin after removal of the paraplast. Slides were examined under a Leica DMLS light microscope with a Canon Power Shot S40 digital camera. Anatomical parameters of leaves were measured using the Leica Q Win measuring program, and statistical analysis of data was performed using STATISTICA 6.0 for Windows.

RESULTS

In all examined species, both obligate and facultative serpentinoephytes, the total quantity of magnesium was always higher than that of calcium. This was the case both in the root and in the stem and leaves. The highest Mg/Ca ratio (2.5) was found in the species Fumana bonapartei, in which magnesium concentration was 1.8, 2.5 and 2.8 times higher than that of calcium in the root, stem, and
leaves, respectively. In addition, the total magnesium concentration was found to be highest (17270 mg kg\(^{-1}\) dw) in this species in relation to all other species studied. A somewhat lower magnesium level and lower magnesium to calcium ratio (1.3) were observed in the serpentine-obligate species *Stachys recta* var. *chrysophaea*. However, in both obligate serpentinophytes, the quantity of magnesium and the Mg/Ca ratio were higher in the aboveground parts of the plant than in the root (Table 1).

In contrast, the facultative serpentinophyte *Seseli rigidum* growing in serpentine soil possesses only a slightly higher quantity of Mg in relation to Ca. It also exhibits a specific distribution of these elements in aboveground and underground organs. A high calcium level was always found in the stem and leaves of this plant, regardless of the substratum (serpentine or calcareous). However, fine differentiation occurs in the root of plants from contrasting habitats. Thus, in the root of plants from serpentine soil, magnesium concentration was almost twice that of calcium, whereas in the root of samples from calcareous soil, the quantity of calcium was as much as 3.5 times higher than that of magnesium (Table 1).

The quantities of iron, chromium, and nickel were considerably higher in the obligate serpentinophytes compared to those in the species *S. rigidum*, regardless of the habitat (serpentine or calcareous) (Table 1).

The highest concentration of iron was found in the root of the species *Stachys recta* var. *chrysophaea* and *Seseli rigidum*, whereas in *F. bonapartei* as much as 55% of the total Fe quantity was accumulated in the stem.

The highest quantity of chromium was also found in the root of all plants examined, being considerable in both obligate serpentinophytes. Conversely, in samples of *Seseli rigidum* from serpentine soil, the chromium level was two times lower, and in plants from a calcareous substratum even seven times lower than that in the obligate serpentinophytes (Table 1).

### Table 1. Concentration of macro- and micronutrients in different organs of obligate and facultative serpentinophytes (mg kg\(^{-1}\) dw).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Mg</th>
<th>Ca</th>
<th>Mg / Ca</th>
<th>Fe</th>
<th>Cr</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fumana bonapartei</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>root</td>
<td>2770</td>
<td>1550</td>
<td>1.8</td>
<td>970</td>
<td>12.47</td>
<td>5.04</td>
</tr>
<tr>
<td>stem</td>
<td>4290</td>
<td>1710</td>
<td>2.5</td>
<td>1760</td>
<td>5.13</td>
<td>28.60</td>
</tr>
<tr>
<td>leaf</td>
<td>10210</td>
<td>3590</td>
<td>2.8</td>
<td>470</td>
<td>4.36</td>
<td>5.51</td>
</tr>
<tr>
<td>Total</td>
<td>17270</td>
<td>6850</td>
<td>2.5</td>
<td>3200</td>
<td>21.96</td>
<td>39.15</td>
</tr>
<tr>
<td><em>Stachys recta var. chrysophaea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>root</td>
<td>3880</td>
<td>3080</td>
<td>1.3</td>
<td>900</td>
<td>10.15</td>
<td>11.33</td>
</tr>
<tr>
<td>stem</td>
<td>4180</td>
<td>2830</td>
<td>1.5</td>
<td>510</td>
<td>5.85</td>
<td>9.98</td>
</tr>
<tr>
<td>leaf</td>
<td>6550</td>
<td>5090</td>
<td>1.3</td>
<td>580</td>
<td>5.86</td>
<td>19.18</td>
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<tr>
<td>Total</td>
<td>14610</td>
<td>11000</td>
<td>1.3</td>
<td>1990</td>
<td>21.76</td>
<td>40.49</td>
</tr>
<tr>
<td><em>Seseli rigidum</em> (serpentine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>root</td>
<td>6410</td>
<td>3520</td>
<td>1.8</td>
<td>570</td>
<td>3.91</td>
<td>13.22</td>
</tr>
<tr>
<td>stem</td>
<td>2460</td>
<td>2610</td>
<td>0.9</td>
<td>70</td>
<td>3.79</td>
<td>5.36</td>
</tr>
<tr>
<td>leaf</td>
<td>5210</td>
<td>7370</td>
<td>0.7</td>
<td>100</td>
<td>2.51</td>
<td>9.10</td>
</tr>
<tr>
<td>Total</td>
<td>14150</td>
<td>13500</td>
<td>1.1</td>
<td>740</td>
<td>10.21</td>
<td>27.68</td>
</tr>
<tr>
<td><em>Seseli rigidum</em> (limestone)</td>
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<td></td>
</tr>
<tr>
<td>root</td>
<td>1860</td>
<td>6770</td>
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<td>340</td>
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<td>3.59</td>
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<tr>
<td>stem</td>
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<td>0.5</td>
<td>60</td>
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</tr>
<tr>
<td>leaf</td>
<td>7360</td>
<td>10150</td>
<td>0.7</td>
<td>70</td>
<td>1.13</td>
<td>5.63</td>
</tr>
<tr>
<td>Total</td>
<td>11280</td>
<td>21110</td>
<td>0.5</td>
<td>470</td>
<td>3.17</td>
<td>10.28</td>
</tr>
</tbody>
</table>
Fig. 1. Herbarium specimen of the obligate serpentinophyte *Fumana bonapartei* (A) and cross section of the stem (B) and leaf (C) with noticeable presence of "vague" contents in parenchymatous cells.

The highest quantity of nickel was found in the aboveground parts of the obligate serpentinophytes. In the species *Seseli rigidum* the Ni quantity was significantly lower, being even as much as 10 times lower in samples collected from a limestone habitat. In samples of this species from serpentine soil Ni accumulated primarily in the root, whereas in samples deriving from calcareous soil the highest nickel level was in the leaves (Table 1).

Leaves of the obligate serpentinophyte *F. bonapartei* are very tiny, ericoid, but of considerable thickness in relation to their surface area (Fig. 1). Epidermal cells are large, with thickened cell walls, and filled with dense contents. They are covered with well developed cuticle. The mesophyll of the plant’s hypostomatic and isobilateral leaves is composed of multilayered palisade parenchyma with thin, barely distinguishable spongy tissue. Vascular bundles are often surrounded by cells containing some dense secreted substances (which turned dark during plant material processing), and sporadic crystals. Parenchyma cells in the bark and pith of the stem are also filled with dense matter, whereas numerous crystal druses are present around vascular bundles (Fig. 1).

On the leaves and stem of *S. recta var. chrysophaca*,

![Diagram](https://via.placeholder.com/150)
there are sparse large glandular trichomes (Fig. 2). The leaves of this obligate serpentinophyte are also hypostomatic and isobilateral in structure. The entire mesophyll is differentiated into several (5-7) layers of relatively short and densely packed palisade cells. The special firmness of the stem of this plant is created by sclerenchyma that occurs discontinuously, forming thick projecting “clusters” in the angles of the squared stem (Fig. 2).

Leaves of the facultative serpentinophyte *Seseli rigidum* are also hypostomatic and isobilateral. They are characterized by well developed cuticle, tiny epidermal cells having thickened external walls, and mesophyll in which palisade tissue is dominant. There are also a number of vascular bundles. The leaves of this plant, particularly of those from serpentine soil, abound in a mass of crystals, mostly around the vascular bundles. The stem of the specimens from serpentine soil are always glaucous, i.e. bluish to violet green (Fig. 3).

![Fig. 3. Herbarium specimens of the facultative serpentinophyte *Seseli rigidum* from serpentine (A) and calcareous (B) soil.](image)

**DISCUSSION**

The results of the present research show that in the plants from serpentine sites the concentration of magnesium is always higher than that of calcium, both in the obligate serpentinophytes and in the facultative ones. Still, the obligate serpentinophyte *Fumana bonapartei* stands out by having a conspicuously high total Mg concentration and high Mg/Ca ratio (17270 mg Mg kg⁻¹ dw in relation to 6850 mg Mg kg⁻¹ dw).
Ca kg$^{-1}$dw). Otherwise, this endemic plant is an indicator plant of serpentine habitats on the Balkan Peninsula, demonstrating that its tolerance to Mg is a heritable, i.e., a constitutive serpentine adaptive trait. Similar Mg tolerance, even unusually high requirements for Mg, was found in some serpentine endemic grasses and other obligate serpentinophytes worldwide (Proctor, 1999).

In contrast, the facultative serpentinophyte *Seseli rigidum* growing on serpentine is well tolerant of a high Mg quantity in the soil, but in such habitats it also takes up calcium sufficiently from the soil and does so on calcareous soil in much greater quantities (almost twice as great). It may be assumed that this plant’s ability to persist in serpentine habitats results from its marked capacity to absorb calcium as an indispensable nutrient.

The analyzed metals – iron, chromium and nickel – present in serpentine rocks of the examined habitats on Mt. Tara are more or less accessible to plants, depending on their solubility at high pH of the soil (Stevanović et al., 2003). The ions of these metals, which might otherwise be toxic to the cells, are retained in varying concentrations in the root, stem, and leaves of the plants examined (Table 1). The total quantity of these elements and their distribution in the plant indicate the ability of plant species to control uptake, exclusion, and/or sequestration of such elements in various plant tissues. In all three plant species from a serpentine habitat (the obligate serpentinophytes and the facultative one) increased quantities of magnesium, iron, chromium and nickel, were found compared to non-serpentine plants. This fact suggests that the geological substratum markedly affects the quantity of some elements in the plant mass, as has been established in other plants on serpentine rocks (Reeves et al., 1983; Kruckeberg, 1984; Becquer et al., 2003; Wenzel et al., 2003; Freites et al., 2004).

Comparatively low uptake of chromium by the plants studied can be explained by its low solubility on this serpentine substratum with relatively high pH values, as has been observed in serpentinophytes from the serpentinized area in Northeast Portugal (Freitas et al., 2004). The highest quantity of chromium was found in the roots of both obligate serpentinophytes and the facultative serpentinicolous species *Seseli rigidum*. This indicates low translocation of chromium to the aboveground organs and its efficient sequestration by roots in these plants. Thus, its harmful effect on a number of physiological processes in the plant (such as respiration, photosynthesis, and enzyme activity) is reduced.

The total quantity of nickel in tissues of all three of the species studied was moderately to extremely high (10-40 mg kg$^{-1}$ dw) in relation to the Ni concentration commonly exhibited by non-serpentine plants (0.1-5 mg kg$^{-1}$ dw). Still, the values characteristic of serpentinophilic Ni-hyperaccumulator plants (primarily some species of *Alyssum*) (Reeves et al., 1999; Wenzel et al., 2003) were not found in the plants analyzed here. Otherwise, in all the plants studied the quantity of nickel was higher in the above-ground parts compared to the root, which can be attributed to easy translocation of nickel in the acropetal direction.

Structural traits of the serpentinophytes studied belong only in part to the complex of the serpentine
syndrome. The obligate serpentinophytes are characterized by a prostrate growth habit, poor branching and tiny leaves that are clearly green in color and xeromorphic in structure. In the mesophyll of isobilateral leaves, palisade tissue and a large number of vascular bundles are dominant, cuticle is strongly developed, and epidermal cell walls are markedly thickened. Such structural characteristics are an adaptive response of the plants to conditions of the habitat, on which is characterized by intense insolation, high air temperatures, shallow soil, and water deficiency in the ground. Multilayered palisade tissue, present on both sides of the leaf in the obligate serpentinophytes and in the species Seseli rigidum from a serpentine habitat, as well as the frequent presence of "vague" contents in epidermal cells (particularly in the species F. bonapartei) greatly contribute to coping with or protection from the intense insolation on such exposed habitats. It may be assumed that the dense "vague" secreted material occurring not only in the epidermis, but also in parenchymatous cells of the leaf and stem of F. bonapartei, derives from substances of secondary metabolism (organic acids or peptides) whose role is to bind toxic ions and prevent their negative effect. A similar mechanism was established in a number of plants that are able to thrive in regions with unusually high levels of heavy metals in soils (Sagnet et al., 1998; Nakazawa et al., 2001; Rai et al., 2004; Kim et al., 2005).

Visible around vascular bundles in the leaf and stem of S. rigidum are exceptionally large crystal druses, particularly in the plants from serpentine soil. Since it is mainly in serpentinocolous specimens that genuine "heaps" of crystal are formed around conducting elements, it may be inferred that sequestration of a large quantity of Mg from the soil is performed in this way. However, it is interesting that the samples of this species on serpentine outcrops in Serbia are very robust plants of considerable height (even as high as over 1 m) and comparatively better developed than the plants from calcareous soil. It may be therefore assumed that adequate Ca absorption and efficient utilization and sequestration of Mg result in excessive development of this plant when growing in serpentine soil.

To be specific, it is well known that Ca and Mg, in addition to their structural role, are also important for regulation of the activity of numerous critical enzymes. This applies particularly to Mg, which is an activator of enzymes of photosynthetic carbon fixation (Hopkins, 1999).

However, plants from serpentine patches deserve more detailed study, which will clarify the physiological–biochemical basis of their adaptations to specialized edaphic conditions. Interestingly, accumulation of some trace elements is thought to benefit serpentinophytes and certain other hyperaccumulators in defense against herbivores or in the general resistance to pathogens (Boyd and Jaffré, 2001). In any case, serpentine areas on the Balkan Peninsula require not only further research, but also adequate protection, given that they are sites of specific plant diversity which are colonized by a small number of unique endemic plants and ones solely adapted to such habitats.

Acknowledgments: This study was financially supported by the Ministry of Science of the Republic of Serbia (Grant No. 143015).

REFERENCES


РАЗЛИКЕ У КОНЦЕНТРАЦИЈИ МЕТАЛА И МОРФО-АНАТОМСКИМ АДАПТАЦИЈАМА ИЗМЕЂУ ОБЛИГАТНИХ И ФАКУЛТАТИВНИХ СЕРПЕНТИНОФИТА ИЗ ЗАПАДНЕ СРБИЈЕ

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У овом раду је испитивана концентрација макро и микро нутријената, Ca, Mg, Fe и Ni, као и количина хрена у органама облигатних серпентинофита Fumana bonapartei и Stachys recta var. chrysophaea које представљају ендемите Балканског полуострва, као и код Seseli rigidum која је широко распрострањена факултативна серпентинофита. Такође, истакнути су структурне адаптивне одлике листова и стабла врста које потичу са серпентинитског као и несерпентинитског подручја планине Таре у западној Србији.