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Zinc (hyper)accumulation in *Cardamine waldsteinii*: from discovery in the herbarium to validation in the field

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ABSTRACT

X-ray Fluorescence Spectroscopy (XRF) is a non-destructive analytical technique that has recently been developed as a tool for high throughput screening for the discovery of trace element hyperaccumulator species in herbarium collections. Herbarium XRF analysis was undertaken of representatives of the Brassicaceae family in the two herbaria in Belgrade (Serbia). This was coupled to field collection and chemical analysis of new samples of *Cardamine waldsteinii*. The data revealed a potentially new zinc hyperaccumulator species, *C. waldsteinii*, with 3050 mg kg^{-1} Zn in its leaves. This compares to 2210 mg kg^{-1} in the leaves of this species collected from the field. These values are below the notional hyperaccumulation threshold for zinc but are nevertheless unusual. The remarkably high accumulation factor for zinc are suggestive of the exceptional potential of *C. waldsteinii* for zinc uptake, even in non-metalliferous, zinc-deficient soils. We recommend following up this study with experimental investigations on *C. waldsteinii* to elucidate its ecophysiology and its zinc tolerance and accumulation characteristics.

Introduction

The detoxification and foliar accumulation of potentially toxic trace elements is one of the three main strategies plants use to cope with anomalous concentrations in the soil (Baker 1981, 1987). In some plants, the extent of this accumulation can be so great that trace element concentrations in leaves are several orders of magnitude higher than in the leaves of surrounding plants growing in the same soils, which classes them as hyperaccumulator plants (Quinn et al. 2010; van der Ent et al. 2013). Intensive studies over the last decades have established the notional values for the trace element concentration in the leaves above which the plant can be considered to be a hyperaccumulator of a particular element. These are: 100 mg kg^{-1} for Cd, Tl and Se; 300 mg kg^{-1} for Cu and Co; 1000 mg kg^{-1} for Ni and As; 3000 mg kg^{-1} for Zn and 10,000 mg kg^{-1} for Mn (van der Ent et al. 2013, 2021). In addition, hyperaccumulator plants typically have higher elemental concentrations in shoots compared to below-ground organs and hence bioaccumulation and translocation factors (shoot-to-root ratio) > 1 (van der Ent et al. 2015). Over 700 hyperaccumulating plant species are currently known globally (Reeves et al. 2018), and most of these species occur in tropical regions, whilst they are under-represented in

temperate regions (Nkrumah et al. 2018). Most of these species hyperaccumulate Ni (~500 taxa; Purwadi et al. 2021), whereas extremely high concentrations of other trace elements are much less common in plants. For example, hyperaccumulation of Cu has been confirmed in just over 50 taxa, hyperaccumulation of Cd in about 10 plant species, and to date, hyperaccumulation of Zn has been found in about 20 taxa (Reeves et al. 2018).

In spite of many new discoveries, hyperaccumulation is a rare phenomenon, occurring in only around 0.2% of angiosperms (Baker and Whiting 2002). However, interest in the discovery of new species is steadily increasing. In this field of research, the last decade has been dominated by the search for potential metal-accumulating species suitable for agromining, i.e. sustainable extraction of certain valuable and recyclable elements, especially Ni (Nkrumah et al. 2021). For Zn, whose deficiency is considered one of the numerous causes of human health problems, there is a constant need for plants with increased content of this element in their edible parts (Palmgren et al. 2008). Considering that, in addition to an extreme uptake of Zn, one of the prerequisites for the biofortification process is the active transport of Zn to the shoot where it is the edible part of the plant, Zn-hyperaccumulating plants could perhaps be successfully

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utilized here. Moreover, since many hyperaccumulating species are restricted to very specific habitat conditions and therefore have a very limited distribution, further studies can contribute to the conservation of such species and their habitats.

In order to discover new hyperaccumulating species as efficiently as possible, high throughput methods of analysis are constantly being developed to enable mass-screening. In the past, a dimethylglyoxime colorimetric test was often used to detect Ni hyperaccumulators, in which a positive reaction (magenta color reaction of the paper impregnated with dimethylglyoxime) reveals high Ni concentrations in the leaf (Chaney et al. 2021). Although this method is less time-consuming compared to classical analyses of plant ionomes (e.g. using ICP-AES, ICP-MS or AAS), its major drawback is that it is only semi-quantitative and only suitable for detecting Ni (Purwadi et al. 2021). In recent years, X-ray fluorescence spectroscopy (XRF) using handheld instruments has increasingly been used as a non-destructive rapid method for screening plant materials enabling the discovery of new hyperaccumulator species (Nkrumah et al. 2018; van der Ent, Echevarria, et al. 2019; Belloeil et al. 2021). Not only this method can be used to scan the plant in the field and analyze a large number of trace elements, but it can also be used to quickly and reliably survey large herbarium collections; i.e. entire genera or families or all representatives from a given geographic area can be systematically scanned and the possible relationship of hyperaccumulation to their phylogenetic status determined (Purwadi et al. 2022). This approach, termed "Herbarium XRF Ionomics", has not only led to confirmation of what is already known, but also to the discovery of a significant number of previously unknown hyperaccumulator species (van der Ent, Echevarria, et al. 2019; van der Ent, Ocenar, et al. 2019; Gei et al. 2020). Thus, analysis of herbarium material from Sabah on the island of Borneo revealed a rather large number of new hyperaccumulator plant species, i.e. 44 hyperaccumulators of Mn, 9 hyperaccumulators of Co, 4 Ni hyperaccumulators, and 2 new Zn hyperaccumulators (especially important, considering that they grow on Zn-deficient soils), while the number of new hyperaccumulators discovered in the New Caledonia herbarium collection is even higher, i.e. 27 Ni hyperaccumulators, 59 Mn hyperaccumulators, 8 Co hyperaccumulators, and 5 taxa that hyperaccumulate Zn (van der Ent et al. 2019). Although XRF scanning of herbarium material greatly facilitates and accelerates the process of discovering new hyperaccumulator species, the data obtained should be carefully examined, especially because of the possible contamination of the material with soil particles. As an additional criterion for a species to be classified as a hyperaccumulator, not only must the element concentrations in the leaves exceed the hyperaccumulation threshold, but these concentrations should also be higher than those in the belowground organs and in the soil (van der Ent et al. 2013). Therefore, the hyperaccumulator status of species with excessive element concentrations detected with the XRF device should additionally be confirmed by laboratory analyses of soil and plant tissue.

The Brassicaceae family is of particular interest for XRF analysis, as more than 100 known hyperaccumulating plant species belong to this family (Reeves et al. 2018) of which 72 taxa hyperaccumulate Ni and 20 Zn, especially in the genera *Noccaea* and *Odontarrhena* (Drozdova et al. 2019). One of the largest genera within the Brassicaceae family is the genus *Cardamine* with 261 recognized species (POWO 2022), distributed across all continents except Antarctica, but mostly in Europe and with a significant proportion of boreal and arctic-alpine species (Carlsen et al. 2009). Similar to several other genera in the Brassicaceae, e.g. *Draba* and *Lepidium*, *Cardamine* is a relatively young genus in which accelerated diversification occurred after it split from the genus *Barbarea*, resulting in a large number of species (Koch et al. 2000; Carlsen et al. 2009). Currently, only a Se -hyperaccumulating species of *Cardamine* is known (Both et al. 2018, 2020). Therefore, the key aim of this study was to perform XRF screening of herbarium specimens of *Cardamine* in Serbian herbaria which hold representative *Cardamine* collections from Europe. Any discoveries were then followed-up with field collection of soil and plant materials for validation.

Material and methods

X-ray fluorescence spectroscopy measurements

X-ray fluorescence spectroscopy (XRF) was undertaken on herbarium specimens of the genus *Cardamine* deposited in two herbaria in Belgrade, Serbia (BEOU and BEO; Thiers 2022). The leaves of a total of 110 specimens of the genus *Cardamine* were analyzed. Most specimens were collected in the Balkan Peninsula (mainly in Serbia, but also in Albania, Greece, Montenegro, Croatia, Romania, and Bulgaria), but a certain number also came from Slovenia, Austria, Switzerland, and France. The instrument used was a Thermo Niton XL5 handheld XRF analyzer (ThermoFisher Scientific, Boston, USA). Herbarium specimens of the genus *Cardamine* were tested in the soil instrument mode for a dwell-time of 30 s. The raw XRF readings for Zn were corrected using an empirical calibration, with the regression formula $y = (0.3766 \, x)^{1.1259}$ ($R^2 = 0.88$) in which y = the calculated corresponding ICP-AES value and x = the measured XRF value (Abubakari et al. 2021).

Analysis of plant and soil samples

Plant samples of *Cardamine waldsteinii* Dyer, a rare and endangered species from western and north-western Serbia (Sabovljević et al. 2022), were collected in May 2022 at the flowering stage for the purpose of a full chemical analysis. The material was collected from a wet meadow in the valley of the Tmuša river in Mt. Jelova Gora (W Serbia) at 835 m a.s.l., in a continental climate zone with an average temperature of 8.33 °C and 906 mm annual precipitation (Fick and Hijmans 2017). Approximately 15 individuals were taken for a bulk sample. The voucher specimen was deposited in the herbarium of the University of Belgrade (BEOU – 17914). After separation into roots and shoots, the plants were carefully washed with deionized water and then air dried. Before digestion in a boiling mixture of HNO_3 and H_2SO_4 (ISO 6636/2 1981), the plants were oven-dried at 85 °C and pulverized.

Atomic absorption spectrophotometry (Shimadzu AA 7000, Kyoto, Japan) was used to determine the concentrations of Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, and Zn in the samples, and all measurements were performed in triplicate. For the series of standard solutions, a 1 g L^{-1} solution of official standards (Carlo Erba, Italy) was used.

Soil samples were collected from the rhizosphere of *C. waldsteinii* individuals. Samples from the same site were pooled together into a composite sample and sieved through a 2-mm sieve. Both actual and exchangeable pH values (pH$_{H2O}$ and pH$_{KCl}$) were measured (in deionized water and in a 1 M KCl solution, respectively) at a solution ratio of 1:2.5 (ISO 10390 1994). To determine pseudo-total concentrations of elements the samples were oven-dried at 85 °C and air-dried for the other element analysis. To assess the percentage of organic matter of the soil samples, a dichromate digestion method was used (FAO 1974). Extractable Ca and Mg were quantified in 1 M ammonium acetate extracts (S/L 1:50) by AAS (van Reeuwijk 2002). Pseudo-total concentrations of Fe, Mn, Ni, Cr, Co, Pb, Zn, Cu and Cd were measured by AAS after digestion of soil samples in a boiling mixture of concentrated HCl and HNO$_3$ acids (ISO 11466 1995), whereas extractable concentrations were measured in 0.05 EDTA extractant (McGrath 1996).

Data analysis

Zinc concentrations in *Cardamine* specimens analyzed are presented as boxplots with median, minimum, and maximum values (in mg kg^{-1}) using Statistica 8 (Statsoft Inc., USA). The availability of trace elements was evaluated as the ratio of EDTA-extractable and pseudo-total concentrations of a given trace element. To estimate accumulation potential of *C. waldsteinii*, bioconcentration (BCF = Croot/Csoil$_a$), bioaccumulation (BAF = Cshoot/Csoil$_a$) and translocation (TF = Cshoot/Croot) factors were calculated.

Results

Elemental concentration in Cardamine herbarium material

Systematic screening of the *Cardamine* herbarium material by the handheld XRF analysis revealed unremarkable concentrations for most of the trace elements (Ni, Cu, Mn, Cd, Hg, Tl) analyzed. However, relatively high Zn values were detected in herbarium specimens of different *Cardamine* species as shown in Figure 1. The mean value of measured Zn in the leaves of the samples analyzed is 386 mg kg^{-1} with absolute Zn concentrations below this value in most species.

The highest concentrations were found in specimens of *C. waldsteinii*, with a maximum value of 3050 mg kg^{-1} in the leaves of specimen collected in 1940 near Tuzla, Bosnia and Herzegovina (BEO, No. 41401; Figure 2). Slightly lower Zn concentration was detected in a specimen from Mt. Zvijezda in Serbia (2740 mg kg^{-1}; BEOU, No. 16270). Considerable variation in Zn concentration was observed among specimens of this species, with an average concentration of 874 mg kg^{-1}. Relatively high concentrations were also found in several specimens of *C. pancicii* Hayek, with 453 mg kg^{-1} as the mean concentration and maximum concentration up to 1600 mg kg^{-1} Zn, respectively. Prior to our study, (hyper)accumulation of Zn in *Cardamine* species was not known. Of the other elements analyzed, Cu was found at a concentration of 6000 mg kg^{-1} in one specimen of *C. pancicii* Hayek. In several other specimens these concentrations were 180–555 mg kg^{-1}, whereas in most they were below the limit of detection. Nickel concentrations in all specimens analyzed were <
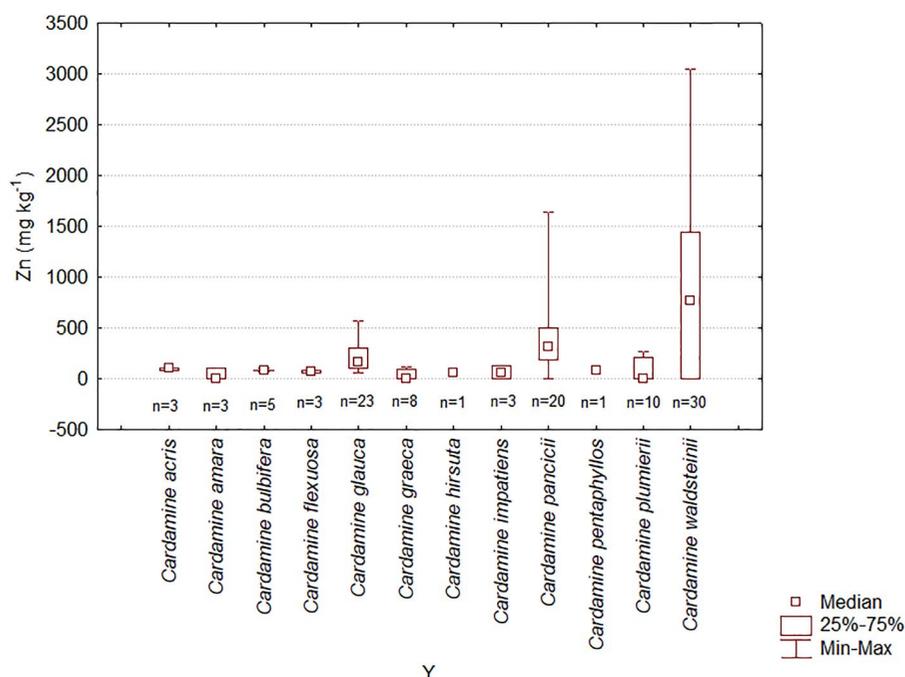

Figure 1. Zinc concentrations in specimens of the genus *Cardamine* deposited at BEOU and BEO herbaria as determined by handheld XRF analysis. *n* = number of specimens included in the analysis.

100 mg kg^{-1} with the highest concentrations found in single specimens of both *C. glauca* Spreng. and *C. plumieri* Vill. (44 and 68.3 mg kg^{-1}, respectively). The concentrations of the other trace elements were low or below the detection limit.

Analysis of C. waldsteinii rhizosphere soil

The results of the chemical analysis of the *C. waldsteinii* rhizosphere soil samples are given in Table 1. The material was collected on siliceous soils in Mt. Jelova Gora (Figure 3),
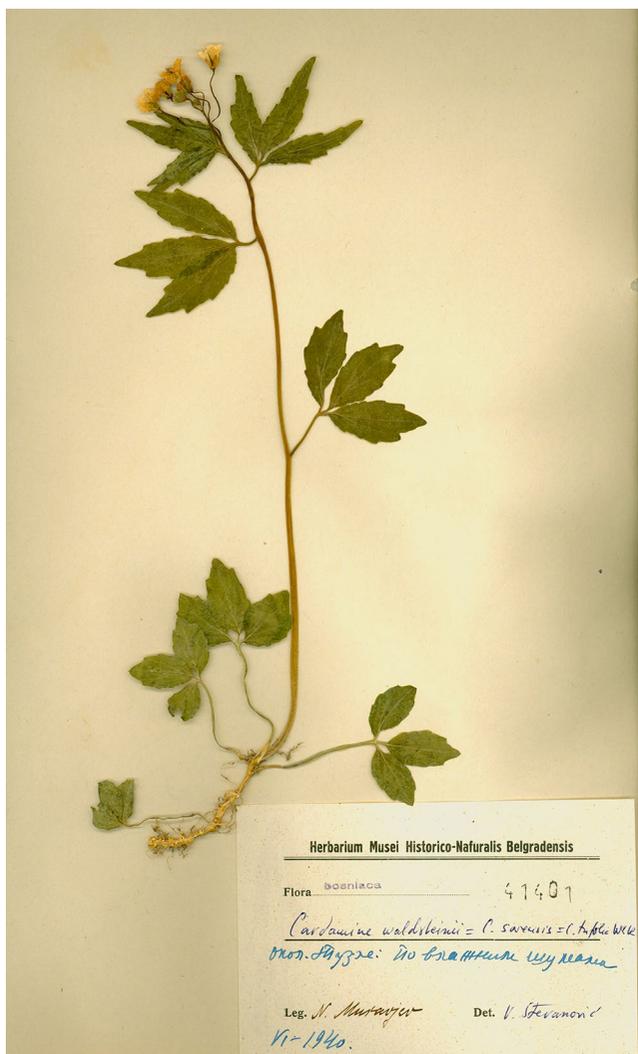

Figure 2. Specimen of *Cardamine waldsteinii* from Bosnia and Herzegovina (near Tuzla) (BEO) with 3050 mg kg^{-1} Zn in the leaves (as measured with handheld XRF).

characterized as strongly acidic and very strongly acidic (5.24 and 4.51 as pH$_{H2O}$ and pH$_{KCl}$ values, respectively) with medium organic content (4.04 ± 0.49% of organic matter). Calcium and magnesium concentrations can be stated as low (1190 and 93.8 mg kg^{-1}, respectively), which is also true for Fe as well as for microelements that can be ranked based on their pseudo-total concentrations, as follows Mn > Zn > Pb > Ni > Cu > Cr > Co > Cd. The bioavailability of the elements is also relatively low, as shown by the ratio between the extractable and the pseudo-total concentrations, with the exception of Cd, where this ratio is 0.992, but at low absolute concentrations.

Analysis of C. waldsteinii plant material

The elemental concentrations in *C. waldsteinii* plant tissues are shown in Table 2. For most trace elements analyzed, these concentrations are notably low, being mostly below < 10 mg kg^{-1} in both the roots and the shoots. An exception is for the concentrations of Zn, the mean values of which in the root and shoot are 1550 and 2210 mg kg^{-1}, respectively. High accumulation factors were found for Zn, especially BCF and BAF (122 and 174, respectively), and for TF also > 1 (Table 3). Values of the factors > 1 were determined for Mn (TF > 1), Cu (BAF and TF > 1), and Cd (BCF, BAF and TF > 1).

Discussion

Previous studies on hyperaccumulation in *Cardamine* taxa focused mainly on Se in *C. violifolia* O.E. Schultz (synonym *C. circaeoides* Hook. f. & Thomson; POWO 2022) (Both et al. 2018, 2020) and *C. hupingshanensis* K.M.Liu, L.B.Chen, H.F.Bai & L.H.Liu (Yuan et al. 2013; Cui et al. 2018; Xiang et al. 2019). At 1965 mg kg^{-1} in the leaves, *C. hupingshanensis* could be considered a hyperaccumulator when growing near Se mine sites in China (Yuan et al. 2013), but given the much higher concentrations in roots (4414 mg kg^{-1}), this hyperaccumulation is likely an "indicator-type" response to extremely high Se exposure rather than true hyperaccumulation. This is similar in *C. enshiensis* (synonym *C. hupingshanensis* according to the authors – Cui et al. 2018. However, the plant name is not known in the relevant databases BrassiBase 2022; POWO 2022; WFO 2022). It had even higher concentrations in plant tissues (2491 mg kg^{-1} in leaves and 2985 mg kg^{-1} Se in roots; Cui et al. 2018). The total concentration of Se in plant tissues of *C. violifolia* was

Table 1. Characteristics of *Cardamine waldsteinii* soil samples: pseudo-total and extractable concentrations of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn (in mg kg^{-1} dw), given as means ± standard deviations for $n = 3$.

Element	Pseudo-total concentration	Extractable concentration	Extractable/Pseudo-total concentration
Cd	0.257 ± 0.006	0.255 ± 0.023	0.992
Co	4.6 ± 0.073	2.28 ± 0.134	0.496
Cr	4.91 ± 1.85	0.378 ± 0.152	0.077
Cu	11.8 ± 0.198	3.8 ± 0.502	0.322
Fe	18,300 ± 546	584 ± 104	0.032
Mn	313 ± 11.4	223 ± 27.5	0.712
Ni	18.5 ± 0.38	5.27 ± 0.38	0.285
Pb	20.1 ± 1.18	9.45 ± 0.885	0.47
Zn	66.1 ± 4.86	12.7 ± 0.86	0.192

Figure 3. *Cardamine waldsteinii* growing in the natural habitat on Mt. Jelova Gora, Serbia – (A) flowers; (B) leaves; (C) plant habitus.

Table 2. Concentrations of Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb and Zn (in mg kg^{-1} dw) in root and shoot sample of *Cardamine waldsteinii*, given as means ± standard deviations for $n = 3$.

Element	Root	Shoot
Ca	606 ± 135	1300 ± 227
Cd	0.445 ± 0.123	1.33 ± 0.388
Co	0.751 ± 0.763	< 0.03
Cr	< 0.05	< 0.05
Cu	3.53 ± 1.35	6.49 ± 1.91
Fe	83.6 ± 25.3	77 ± 26.8
Mg	952 ± 172	1440 ± 206
Mn	12.3 ± 3.01	24.3 ± 6.69
Ni	2.76 ± 0.89	1.96 ± 0.96
Pb	6.76 ± 1.73	4.3 ± 1.91
Zn	1550 ± 248	2210 ± 312

Table 3. Bioconcentration (BCF), bioaccumulation (BAF) and translocation (TF) factors calculated for trace elements in the samples of *Cardamine waldsteinii*.

Element	Bioconcentration factor	Bioaccumulation factor	Translocation factor
Cd	1.75	5.22	2.99
Co	0.33		
Cr	–	–	–
Cu	0.93	1.71	1.84
Mn	0.06	0.11	1.98
Ni	0.52	0.37	0.71
Pb	0.71	0.46	0.65
Zn	122	174	1.43

3.7 g kg^{-1}, with no data reported on concentrations in individual plant parts (Both et al. 2018, 2020). There are also preliminary data on the hyperaccumulation of Cd in *C. hirsuta* L. Up to 142 mg kg^{-1} Cd has been detected in above-ground parts of this species (Lin et al. 2014), exceeding the notional hyperaccumulation threshold (100 mg kg^{-1}; van der Ent et al. 2013), but under experimental conditions with a TF < 1, it suggests an "indicator-type" response and not hyperaccumulation.

The XRF analyses of herbarium material in this study revealed a potentially new (hyper)accumulator species within the genus *Cardamine*, *C. waldsteinii*, exceeding the notional threshold for hyperaccumulation of 3000 mg kg^{-1} in one specimen. Although the Zn concentrations detected in other specimens are below this threshold, most exceed 1000 mg kg^{-1}, with a mean concentration of 1250 mg kg^{-1} in specimens with detectable Zn. XRF analysis of herbarium material of *C. waldsteinii* deposited in the Natural History Museum in Paris (MNHN), revealed even higher Zn concentrations (3780 mg kg^{-1}; No. P06806513) in a specimen from Croatia (unpublished).

To confirm the hyperaccumulation potential of *C. waldsteinii*, corresponding soil and plant samples were collected from the field. In addition to the high average Zn concentration found in the plant shoots (Table 2), the exceptional ability of this species to accumulate Zn is suggested by the accumulation factors, considering that extremely high values for BCF and BAF were recorded with TF > 1 (Table 3), despite the generally observed tendency of plants to retain Zn in the root, with weak translocation of the element to the shoot (Gupta et al. 2016). Accordingly, the gradient of the average Zn concentration in the soil, root and shoot is 12.7 < 1550 < 2210 mg kg^{-1}. The strong shoot-to-root ratio, with exceeding the hyperaccumulation threshold in non-metalliferous soils has been previously reported in *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz (53,900 mg kg^{-1} in leaves; Stein et al. 2017) and in two subspecies of *Dichapetalum gelonioides* (Roxb.) Engl. (*D. gelonioides* subsp. *pilosum* Leenh. and *D. gelonioides* subsp. *sumatranum* Leenh.; with the foliar Zn concentration of 26,360 and 15,660 mg kg^{-1}, respectively; Baker et al. 1992) living exclusively in Zn-deficient soils. In addition to the distinct internal potential, Zn accumulation in *C. waldsteinii* shoots was also promoted by soil pH, i.e. strongly acidic conditions (pH$_{H2O}$=5.24; Soil Survey Division Staff 1993),

as it is known that lowering pH greatly increases the extractable concentration of Zn in soils (Wang et al. 2006; Gupta et al. 2016).

The concentrations of the other trace elements are much lower (Table 2). Even for Mn, Cu and Cd, for which accumulation and translocation factors > 1 were determined, the concentrations in plant tissues are quite low because of their low concentrations in the soil and are several-fold lower than the hyperaccumulation thresholds. For Fe, Ni, Pb and Co, not only low absolute concentrations were found, but also low accumulation factors. Apart from *C. waldsteinii*, relatively high Zn concentrations were found in three specimens of *C. pancicii* (Figure 1). Of particular interest were the Cu concentrations, where the maximum value of 6000 mg kg^{-1} was well above the hyperaccumulation threshold (300 mg kg^{-1}; van der Ent et al. 2013). However, the most likely cause of such high concentrations is soil contamination, especially considering that *C. pancicii* is a steno-endemic species of the Suvo Rudište peak on Mt. Kopaonik in Serbia (Lakušić et al. 2006), which is known to be the site of an abandoned Fe-Cu mine (Vukoičić et al. 2020). Previous results already indicated the anomalous Cu content in the soil from this site (4360–4710 mg kg^{-1}; Tomović et al. 2018; Jakovljević et al. 2021).

Conclusions

Our results clearly show that *C. waldsteinii* has highly anomalous Zn concentrations and has the potential to be a Zn hyperaccumulator. Further analysis of material from the field is described, but the sampling was limited in order not to compromise rather small population size of this rare species. It would be worthwhile to undertaken experimental studies on *C. waldsteinii* grown from seed to elucidate its Zn tolerance and accumulation responses under controlled conditions. This study highlights the usefulness of the XRF technique in the discovery of new hyperaccumulator species, especially for elements not frequently studied.

Acknowledgments

Authors' contributions

KJ, TM, and GE created a concept and collected the data; KJ prepared the original draft; GA performed the laboratory analyses; TM, AVDE, AJMB, GT, and GE discussed the results and contributed to the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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