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RESEARCH ARTICLE

Concentration of some metals in soil and plant organs and their biochemical profiles in *Tulipa luanica*, *T. kosovarica* and *T. albanica* native plant species

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Abstract The purpose of this study was to determine the concentration of some metals (Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn, Ca and Mg) in soil of serpentine and limestone sites, their bioaccumulation and impact on some biochemical parameters in *T. luanica*, *T. kosovarica* and *T. albanica* plants. *T. kosovarica* and *T. albanica* grows in serpentine soil, while *T. luanica* grow in limestone soil. The research showed that concentrations of Cd, Co, Cr, Fe, Mn and Ni were significantly higher at serpentine soil sites in comparison with limestone sites, while concentrations of Pb, Cd, Co and Cr in bulbs, leaves and seeds were under the limit of detection. The concentration of Ni in plant samples of *T. kosovarica* was significantly higher in comparison with its concentration in *T. albanica*, but it was under the limit of detection in *T. luanica*. Moreover, concentrations of Al and Fe in leaves of *T. kosovarica* and *T. albanica* were higher in comparison with *T. luanica*. The concentration of Mg was significantly higher in *T. kosovarica* and *T. albanica* than in *T. luanica.* The δ-aminolevulinic acid dehydratase activity, malondialdehyde and glutathione contents in leaves of *T. luanica* were higher in comparison with *T. kosovarica* and *T. albanica*. In addition, the amounts of total chlorophyll and δ-aminolevulinic acid (ALA) in leaves of *T. albanica* were higher in comparison with *T. kosovarica* and *T. luanica.* Our findings show that target organs of metal accumulation in three Tulip species appears to be leaves > seeds > bulbs, while the biochemical parameters show that limestone sites represent a less stressful habitat for growing these plant species in comparison with serpentine sites.

Keywords Tulipa · Metals · δ-Aminolevulinic acid dehydratase · Malondialdehyde · Glutathione

Abbreviations

ALA-D (EC 4.2.1.24)	δ-Aminolevulinic acid dehydratase
ALA	δ-Aminolevulinic
MDA	Malondialdehyde
GSH	Reduced glutathione
PGB	Porphobilinogen
TBA	Thiobarbituric acid
Al	Aluminum
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
Fe	Iron
Mn	Manganese
Ni	Nickel
Pb	Lead
Zn	Zinc
Ca	Calcium
Mg	Magnesium

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Introduction

The genus *Tulipa* is comprised of about 100 species (Bryan 2002). The species are widely spread from southern Europe, northern Africa, the Middle East, and central Asia including China. Most of the native tulip species are distributed in Iran (Ghahreman et al. 2007) and fewer are distributed in the Balkan Peninsula (Govaerts 2010). In this case, in Kosovo, five native tulipa species have been recorded: *Tulipa australis, T. serbica, T. gesneriana subsp. T. scardica, T. kosovarica* and *T. luanica* (Millaku and Elezaj 2015; Shuka et al. 2010, 2012). The number of species of this genus increased in Kosovo after the discovery of two new species *Tulipa kosovarica* by Shuka et al. (2012) and *Tulipa luanica* by Millaku and Elezaj (2015), while in Albania, the species *Tulipa albanica* was discovered by Shuka et al. (2010). *T. kosovarica* and *T. albanica* grow in serpentine soil, while *T. luanica* grows in limestone soil.

Serpentine soils are formed by weathering of ultramafic rocks and are characterized by a low calcium to magnesium ratio and high concentrations of iron, nickel, magnesium, cobalt and chromium. These substrates contain heavy metals toxic for the environment (Herath et al. 2014); they are also often deficient in essential nutrients such nitrogen, potassium and phosphorus (Anacker 2014). In Europe, the largest serpentine areas are in the Balkan Peninsula, including Kosovo and south-eastern Albania. Serpentine soils are very important for the special flora and for diversity of plant species; generally they are poor in vegetation but very rich in rare and endemic species.

There are few studies focussing on serpentine soils generated heavy metal stress adaptations of plants (Brady et al. 2005). Serpentine substrates by their composition have many disadvantages to the growth and development of many plant species; but some serpentine plant species during evolutionary development have developed biochemical and physiological adaptations, especially selectivity and tolerance to many metals such as Ni, Mg and Ca including their transfer and distribution in leaves (Vicic et al. 2014). For this reason, serpentine substrates are very suitable for assessing a plant's ability to adapt to these conditions and interactions with them. Essential and nonessential heavy metals that are found in serpentine soils generally have toxic effects on the plant, that situation being reflected in decreasing biomass, chlorosis, growth inhibition, water balance, DNA synthesis, etc. (Singh et al. 2016). The heavy metals in toxic concentrations affect, among other things, some metabolic cell processes with a particular emphasis on the pathway of chlorophyll biosynthesis (Bertrand and Poirier 2005) which is an important physiological process in green plants and is regulated at several steps (Beale 1999). In line with this, it is important to study the role of the enzyme δ-aminolevulinic acid dehydratase (ALA-D) in earlier steps of the pathway, because it's activity is fundamental for biosynthesis of tetrapyrroles such as porphyrins, hemes, and chlorophyll (Jaffe 2000). ALA-D catalyzes the asymmetric condensation of two molecules of δ-aminolevulinic acid (ALA) to porphobilinogen and is sensitive to metals due to its sulfhydrilic nature (Rocha et al. 1995). Divalent and monovalent cations are utilized by ALA-Ds metalloenzymes belonging to different sources (Jaffe 2000). Some heavy metals having high concentration in plants can inhibit ALA-D activity, thereby increasing the amount of ALA (Gupta et al. 2013). Furthermore, ALA accumulation in the chlorophyll and heme pathway leads to generation of reactive oxygen species (ROS) as well as cellular oxidative stress (Noriega et al. 2007). The development of oxidative stress symptoms from biotic or abiotic factors causes oxidative damage to lipids. Malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, is a common marker of oxidative stress and the antioxidant status in animal and plant organisms (Auer et al. 1995). Cells are able to act against free radicals with the help of various antioxidant system developed as a part of defense system. Glutathione (GSH) is considered one of the most important antioxidant metabolites and represents the first line of defense against reactive species. GSH plays a major role in protecting cells against oxidative stress (Hasanuzzaman et al. 2017).

There has been no research conducted on the concentration of metals and ALA-D activity, ALA, chlorophyll content, MDA and GSH for any plant species from genus Tulipa. It is known that serpentine soils are enriched with metals compared with limestone soils. Since, *T. kosovarica* and *T. albanica* grow on serpentine soils while *T. luanica* grows in limestone soil, it will be important to determine that: (a) are these plant species metal hyperaccumulators; (b) what is the effects of metal accumulation on the different pathway steps of chlorophyll biosynthesis and on level of oxidative stress; and (c) what are the differences between these plant species. The results of this research will provide more information on the mechanisms involved in the plant tolerance and adaptation in these endemic plant species.

Material and methods

Soil and plant sampling

The investigation was carried out in two serpentine regions (Surroi in Albania and Mrasor in Kosovo) and in one limestone region (Pashtrik in Kosovo). Soil was collected from the upper horizon at a depth of approximately 15 cm

and in the location of plant sampling. Soil samples were air-dried and mechanically sieved to < 2 mm particle size prior to analysis. The plant samples were collected from their sites: *Tulipa albanica* at Surroi locality (Albania), *Tulipa kosovarica* at Mrasor locality (Kosovo) and *Tulipa luanica* at Pashtrik locality (Kosovo). More than 30 plant samples from each plant species were collected from their habitat; bulbs, leaves and seeds were washed in de-ionised water, dried at 80 °C for 48 h and ground to a fine powder.

Soil properties

Soil pH was measured in H_2O suspension of soil and 0.01 M $CaCl_2$ with a ratio of 1:2.5 (DIN ISO 10390 2005). The total amount of organic matter (OM) was determined by the ignition method. Particle size distribution was determined by a combined sieving and pipette method after decomposition of carbonates (HCl) and organic matter (H_2O_2) and dispersion in Na-pyrophosphate (DIN EN ISO 14688-1:2003-01 2003).

Metal analysis

Soil and plant samples were mineralized with a microwave digester (CEM - Mars 6). Plant samples (500 mg) were digested by adding 8 mL HNO_3 and 2 mL H_2O_2. Soil samples (500 mg) were digested by adding 9 mL HNO_3 and 3 mL HCl. Solutions were collected in flasks and adjusted to 25 mL with distilled water. Concentrations of Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn, Ca and Mg were determined by ICP-OES spectrometer (Sepctro&Ametek—Spectroblue) at Namik Kemal University, Tekirdag, Turkey.

Sample preparation and biochemical assay

Biochemical analyses were carried out with fresh leaves of the three tulip species. Leaves were collected during flowering time from the natural habitat, and all steps from sample collection to preparation were conducted at a controlled temperature (4 °C).

δ-Aminolevulinic acid dehydratase (ALA-D) activity

The enzyme was assayed colorimetrically using modified Ehrlich reagent to estimate the amount of PBG (porphobilinogen) formed. Extraction and assay of ALA-D was carried out according to the procedure described by Jain and Gadre (2004). The enzyme was extracted with ice-cold 50 mM Tris–HCl buffer (pH 8.4) containing 0.2% Triton X100. The fresh leaves (1000 mg) of each tulip species were ground to a fine powder under ice-cold temperature in a mortar. Immediately after grinding, 1 g of polyvinylpolypyrrolidone was mixed with the powdered tissue to prevent phenolic oxidation. After filtration, the homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C. The pellet was discarded, and the supernatant was used as the enzyme source. One mL of enzyme extact was incubated with 0.27 mL of 1 mg mL^{-1} ALA, 1.35 mL of 50 mM Tris–HCl buffer (pH 8.5) and 0.08 mL of 0.02 M $MgCl_2$. The reaction was initiated by adding the extract at time zero. After 1 h of gentle shaking (150 rpm) at 37 °C, the reaction was stopped by adding 0.3 mL of 3 M TCA, followed by centrifugation at 5000 × g for 10 min. For PBG estimation, the supernatant was mixed with Ehrlich reagent (prepared fresh by dissolving 1.0 g of 4-dimethyl aminobenzaldehyde in 30.0 mL of glacial acetic acid and 8.0 mL of 70% PCA, and then made up to 50.0 mL with glacial acetic acid) in a ratio of 1:1 (v/v); absorbance was measured at 553 nm after 15 min against zero time control. One unit of enzyme activity was defined as 1 nmol of PBG formed per hour. Protein content was estimated by the method of Lowry et al. (1951).

Aminolevulinic acid (ALA) content

This was determined according to the method of Tewari and Tripathy (1998). Fresh leaves (500 mg) of each tulip species were homogenized using 1.0 mL of 1 M Na-acetate buffer (pH 4.6). After centrifugation at 15,000 × *g* for 15 min at 4 °C, ALA of the supernatant was condensed into PBG using ethylacetoacetate: 0.7 mL supernatant, 0.8 mL distilled water and 0.1 mL ethylacetoacetate mixture were kept in a boiling water bath for 10 min. After cooling, an equal volume of Ehrlich reagent was added and the colored complex formed was read for absorbance at 553 nm. Amount of PBG formed was calculated using a standard curve of ALA and the results were expressed in μM ALA g^{-1} fresh leaf weight (FW).

Total chlorophyll

Chlorophyll was extracted from fresh leaves (100 mg) of each tulip species with 80% acetone. Chlorophyll contents were calculated using absorbance values at 663 nm and 645 nm measured by a UV–vis spectrophotometer. The new extinction coefficients and re-evaluated equations of Lichtenthaler were applied (Porra et al. 1989). Total chlorophyll content was expressed as mg g^{-1} fresh leaf weight (FW).

Glutathione (GSH) assay

Fresh leaves from each tulip species were homogenized with 5% TCA. The homogenate was centrifuged at 15,000 × *g* for 20 min and the pH of the supernatant was adjusted to 4.0–5.0 with 1 M NaOH. The content of

glutathione (GSH) in crude extract was determined using the Ellmann [DTNB: 5,5′-dithiobis (2-nitrobenzoic acid)] procedure (Primiano and Novak 1992), in which the reaction mixture comprised 0.1 mL of sample, 2 mL of 100 mM pH 8.4 Tris–HCl buffer and 0.1 mL Ellmann reagent (60 mg/100 mL Tris–HCl buffer 0.1 M, pH 7.0). Absorbance of the reaction mixture was read at 412 nm. Glutathione concentration in the samples was calculated from the standard curve of GSH. The data were expressed as μM g^{-1} fresh weight of leaf.

Malondialdehyde (MDA) assay

Lipid peroxidation was estimated by determining malondialdehyde (MDA) contents in leaves according to the method of Hodges et al. (1999). Fresh leaves (500 mg) from each tulip species were ground in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C. MDA levels were estimated according to the corrected thiobarbituric acid (TBA) method. Two milliliters of extraction solution and 3 mL 0.5% TBA in 10% TCA were mixed vigorously. The mixture was heated at 95 °C in a constant temperature water bath for 30 min and then cooled on ice to room temperature. After centrifuging at 5000 × *g* for 10 min, the supernatant absorbance at 450, 532 and 600 nm was measured using a UV–vis spectrophotometer. The concentration of MDA was determined using the formula C_{MDA} (μmol mL^{-1}) = 6.45 × (D532–D600) − 0.56 × D450, where D450, D532 and D600 are the absorbencies at 450, 532 and 600 nm, respectively. The data were expressed as μM g^{-1} fresh weight of leaf.

Data analysis

Statistical analysis of the results was carried out with Sigma stat 32 programs 2004 STAT Software. The data presented in the paper represent the average of at least four independent experiments with ± S.E. For each continuous variable, a distribution form was determined, and significant differences between means were checked by Student's *t* test. The Bioaccumulation factor (BF) was calculated to determine the degree of metal accumulation in the plants grown at the serpentine and limestone sites, using following equation:

$$BF = \frac{C_{plant}}{C_{soil}}$$

where C_{plant} is the average of concentration of an element in the bulbs and leaves and C_{soil} is the total concentration of the same element in the soil where the plant was grown.

Results and discussion

Soil properties and metal concentrations

The pH in H_2O of the serpentine soil samples (Mrasor and Surroi) were slightly acidic, whereas in limestone soil (Pashtrik), pH was around neutral (Table 1). This is in agreement with a previous study which showed that pH of serpentine soils varied from moderately acidic to slightly alkaline (Bani et al. 2010). In our study, the percentage of organic matter in limestone soil was moderate (18.30%) but in both serpentine soils were low (around 12%). Slit and clay in serpentine soil samples (Marsor and Surroi) were very low in comparison with limestone soil samples (Pashtrik), on other hand, the percentage of sand in limestone soil samples were lower in comparison with serpentine soils. Our results for soil texture of serpentine soils are in accordance with other studies. In this case, Brady et al. (2005) reported that slit and clay contents in serpentine soils are generally minimal.

Concentrations of Cd, Co, Cr, Fe, Mn and Ni were significantly higher in serpentine soil sites in comparison with the limestone site, while concentrations of Al, Cu, Pb and Ca were higher in the limestone site in comparison with serpentine sites (Table 2). Our results concerning higher concentrations of Co, Cr, Ni and Mg in serpentine sites relative to the limestone site are consistent with results of Shallari et al. (1998), which also established high concentrations of Ni, Cr and Co in serpentine sites in several regions in Albania. High concentrations of Fe, Ni and Cr were observed only in serpentine soils compared with other soils (Anacker 2014; Rabchevsky 1985). Based on our results, at serpentine sites the concentration of Co varied from 130 to 140 mg kg^{-1} DM, Cr varied from 400 to 550 mg kg^{-1} DM and Ni varied from 1500 to 1900 mg kg^{-1} DM. Bani et al. (2013) observed similar results, in which the total concentrations of Co, Cr and Ni at all serpentine sites were high and ranged from 62 to 260 mg kg^{-1} for Co, 178 to 1035 mg kg^{-1} for Cr and 1658 to 3077 mg kg^{-1} for Ni.

Serpentine soils were also rich in Mg, but deficient in Ca. The lowest concentration of Mg was established in the limestone site (0.29%) and the highest in both serpentine sites (2.20; 2.78%), while the Ca concentration was lowest in both serpentine sites (0.19; 0.52%) and highest in the limestone site (1.15%). The Mg/Ca ratio in both serpentine soil sites was higher (5.35; 11.56) in comparison with the limestone site (0.25). Similarly, a higher ratio of Mg/Ca was recorded in serpentine soils at different sites by other researchers (Shallari et al. 1998; Bani et al. 2013).

Table 1 Physico-chemical properties of the soil at experimental site

Sites	pH (water)	pH ($CaCl_2$)	OM%	Soil texture in %			Texture name (class)
				Sand	Silt	Clay	
Pashtrik	7.18	6.60	18.30	74.50	9.70	15.80	Sandy loam (SL)
Mrasor	6.89	6.45	12.06	86.10	3.80	10.10	Loamy sand (LS)
Surroi	6.63	6.11	12.97	86.20	3.20	10.60	Loamy sand (LS)

OM organic matter

Concentration of metals in plant organs

Concentrations of Al, Cu, Fe, Mn, Ni, Zn, Ca and Mg in bulbs, leaves and seeds of *T. luanica, T. kosovarica* and *T.albanica* are presented in Table 3. The concentrations of Al and Fe in bulbs and leaves of *T. kosovarica* and *T.albanica* were higher compared with *T. luanica*. On the other hand, the Cu and Mn concentrations in leaves and seeds of *T. luanica* were significantly higher ($P < 0.001$) compared with *T. kosovarica* and *T. albanica*. The concentration of Ni in bulbs, leaves and seeds of *T. kosovarica* was significantly higher compared with concentration in *T. albanica*, while in *T. luanica, it* was under the limit of detection at ppb and ppm levels by ICP-OES. The highest concentration of Ni in serpentine tulips (*T. kosovarica* and *T. albanica*) can be explained as an evolutionary adaptation of these plants in the Ni-rich serpentine soil environment. The Ni concentration in plant organs (*T. kosovarica* and *T. albanica*) was in the order of seeds > leaf > bulb; this order of concentration can be indicative of a useful mechanism by plants for excretion of Ni as a toxic metal. Toxicity due to Ni can be overcome by a mechanism of its sequestration in leaves apart from Ni exclusion mechanism (Yusuf et al. 2011). Higher Ni concentrations in reproductive organs is observed in species not affected by serpentine soils whereas similar concentrations of Ni was observed in all organs of endemic and indicator species, indicative of the absence of any mechanism restricting Ni accumulation in reproductive organs in the plant species not associated regularly with serpentines (Meindl et al. 2014).

Concentrations of Pb, Cd, Co and Cr in bulbs, leaves and seeds in all examined plant organs were under the limit of detection. It is well known that Pb entrance into plants is mainly through the roots via the apoplast pathway or calcium ion channels. Once located in the roots, Pb tends to sequester in root cells. Only a limited amount of Pb is translocated from roots to shoot tissue because there are natural plant barriers in the root endodermis (e.g. casparian strips). Pb mainly accumulates in the roots (ca 93–96% of Pb uptake) and hypocotyls (ca 4–6%), whereas only trace Pb amounts are found in cotyledons growing in solution with 10^{-3}M Pb (Burzynski 1987).

Our results showing higher concentrations of Ni, Al, Fe and Mg in plant samples of serpentine tulips (*T. kosovarica* and *T. albanica*) are consistent with results of several authors (Shallari et al. 1998; Bani et al. 2013), who also reported higher concentrations of the above mentioned elements in different plant family species (*Brassicacea, Charyophyllacea, Astereacea, Poaceae*, etc.) from serpentine sites in Albania. Altinozlu et al. (2012) have reported significant differences in Ni content among some hyperaccumulator Brassicaceae plant species and other species from this family endemic to Turkey.

Our results reveal significantly higher Mg concentrations ($P < 0.001$) in all analyzed plant samples of *T. kosovarica* and *T. albanica* compared with *T. luanica*, while the concentration of Ca in plant organs was significantly higher ($P < 0.001$) in *T. luanica* compared with *T. kosovarica* and *T. albanica* (Table 3). The results of this research indicate that higher concentrations of Mg in serpentine tulips and Ca in limestone tulips are in accordance with results of other authors dealing with this field of expertise (Shallari et al. 1998; Brady et al. 2005; Wislocka et al. 2006; Bani et al. 2007), who have found higher concentrations of Mg and low concentrations of Ca in hyperaccumulator plants from serpentine sites. This higher ratio of Mg/Ca in plants growing in serpentine soils has also been reported by these authors.

Bioaccumulation factor (BF) of some metals such as Al, Cu, Fe, Mn, Ni and Zn in *T. luanica*, *T. kosovarica* and *T. albanica* are presented in Fig. 1. The results show that value of BF for all metals was less than one. The results of BF suggest that these plants species are not hyperaccumulator plants. On the other hand (Bani et al. 2010; Shallari et al. 1998), established BF values more greater than one in hyperaccumulator plants (*Alyssum* and *Thlaspi*) from serpentine soil.

Biochemical profile

The results of ALA-D activity and levels of ALA, total chlorophyll, MDA and GSH in *T. luanica, T. kosovarica* and *T. albanica* are presented in Table 4. Enzyme activity for ALA-D was significantly higher ($P < 0.001$; $P < 0.003$, respectively) in leaves of *T. luanica* and *T.*

Table 2 Metals, Ca and Mg concentrations in soil of two serpentine sites (Mrasor and Surroi) and a limestone site (Pashtrik)

Sites		mg kg^{-1} DM										% DM		Ratio
		Al	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	Ca	Mg	Mg/Ca
Pashtrik		18,770 ± 680	0.6 ± 0.1	24 ± 2.2	127 ± 16	31 ± 1.4	42,892 ± 3094	1277 ± 66	152 ± 24	39 ± 9.2	77 ± 16	1.15 ± 0.12	0.29 ± 0.02	0.25
Mrasor		12,798 ± 995	3.9 ± 0.2	134 ± 6.1	415 ± 40	27 ± 3.9	59,699 ± 4249	1659 ± 50	1531 ± 120	21 ± 5.1	53 ± 7.7	0.52 ± 0.11	2.78 ± 0.34	5.35
Surroi		11,618 ± 603	4.0 ± 0.4	137 ± 19	516 ± 40	15 ± 2.3	61,884 ± 5454	1700 ± 129	1870 ± 149	22 ± 5.2	85 ± 10	0.19 ± 0.02	2.20 ± 0.25	11.56
Significance	P:M	< 0.001	< 0.001	< 0.001	< 0.001	< 0.007	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	
	P:S	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.013	< 0.001	< 0.001	
	M:S	< 0.001	= 0.367	= 0.481	< 0.001	< 0.001	= 0.231	= 0.361	< 0.001	= 0.934	< 0.001	< 0.001	< 0.001	

DM dry mass, *P* Pashtrik, *M* Mrasor, *S* Surroi

The data are presented by mean and standard error (±). Means in each column followed by same letters are not significantly different at *P* 0.05 and *P* 0.001

kosovarica compared with *T. albanica*. A positive significant association was established between concentrations of Al, Ca, Cu, Fe and Mg and ALA-D activity in leaves of *T. luanica*, while a negative correlation was recorded between Cu, Fe, Mn and Ni and ALA-D activity in *T. kosovarica* and *T. albanica* (Table 5). The higher activity of ALA-D in leaves of *T. luanica* may be a result of hormesis effect of the above metals, especially of lower concentration of iron in this plant. We presume that inhibition of ALA-D activity in leaves of *T. kosovarica* and *T. albanica* is a result of higher concentration of Fe (0.7 and 3.0 time higher, respectively) in these two serpentine tulips. It is well known that lower concentrations not only of essential metals as well as toxic metals stimulate the activity of different enzyme, while the higher concentration even of essential metals have inhibitory effect.

The negative correlation between Cu concentration and ALA-D activity in the leaves of *T. kosovarica* and *T. albanica* can be as a consequence of synergetic effect of Cu and Ni, since the Ni concentration in *T. luanica* leaves was under the limit of detection. Our results are consistent with results of Ghasemi et al. (2009), showing toxic effects in *Alyssum inflatum* plant experimentally exposed in medium with Cu and Ni supplementation in contrast to medium only with Cu.

Inhibition of ALA-D activity in leaves of *T. kosovarica* and *T. albanica* may be a result of higher concentrations of Al, Fe and Ni in leaves, since ALA-D activity was inhibited not only by Pb but also by higher concentrations of Al and Ni. Our results of ALA-D activity inhibition are consistent with results of Pereira et al. (2006), in which cucumber (*Cucumis sativus*) plants experimentally exposed to different aluminium salt $Al_2(SO_4)_3$ doses established remarkable inhibition of ALA-D activity. The results of ALA-D activity inhibition by Ni in leaves of *T. kosovarica* and *T. albanica* are consistent with results of Manankina et al. (2003), where algae (*Euglena gracilis*) incubated with higher concentrations of Ni markedly inhibited chlorophyll accumulation and ALA-D activity as compared to control cells. At this concentration, Ni also inhibited heme biosynthesis and strongly stimulated ALA production.

Several authors found inhibition of ALA-D activity in different plant species such as cucumber (Gonçalves et al. 2009) and maize (Sarangthem et al. 2011; Gupta et al. 2013) experimentally exposed to Cd and Hg, respectively. Morsch et al. (2002) reported inhibition of ALA-D activity in radish leaves when exposed in vitro and in vivo to Pb, Zn, Cd and Hg.

Total chlorophyll content was slightly higher in *T. albanica* (9.09 mg g^{-1} FW) and *T. kosovarica* (8.86 mg g^{-1} FW) compared with *T. luanica* (8.33 mg g^{-1} FW). Higher total chlorophyll content in serpentine tulips may be a result of the higher concentration of Mg and Fe in

Table 3 Metals, Ca and Mg concentrations in bulb, leaf and seeds of *T. luanica*, *T. kosovarica* and *T. albanica*

			Species			Significance		
			T. luanica Pashtrik	*T. kosovarica* Mrasor	*T. albanica* Surroi	TL:TK	TL:TA	TK:TA
mg kg^{-1} DM	Al	Bulb	4.96 ± 0.89	7.59 ± 1.26	14.30 ± 1.22	< 0.001	< 0.001	< 0.001
		Leaf	57.45 ± 6.92	77.35 ± 13.22	66.62 ± 10.55	< 0.001	= 0.251	= 0.206
		Seed	7.76 ± 0.49	2.52 ± 1.61	6.70 ± 0.31	< 0.001	= 0.005	< 0.001
	Cu	Bulb	3.09 ± 0.11	3.47 ± 0.07	2.68 ± 0.36	< 0.001	= 0.058	< 0.001
		Leaf	7.99 ± 0.39	5.83 ± 1.24	7.03 ± 0.48	< 0.001	< 0.001	= 0.133
		Seed	10.09 ± 0.30	5.81 ± 0.33	7.29 ± 0.36	< 0.001	< 0.001	< 0.001
	Fe	Bulb	25.03 ± 2.27	47.27 ± 3.09	35.20 ± 1.44	< 0.001	< 0.001	< 0.007
		Leaf	94.66 ± 7.35	131.60 ± 28.00	283.60 ± 99.08	< 0.001	< 0.001	< 0.001
		Seed	44.40 ± 8.27	32.65 ± 2.41	66.60 ± 3.29	< 0.001	< 0.001	< 0.001
	Mn	Bulb	2.37 ± 0.28	2.89 ± 0.25	2.40 ± 0.35	< 0.008	= 0.861	< 0.003
		Leaf	20.95 ± 2.23	18.27 ± 1.41	15.70 ± 0.87	< 0.008	< 0.001	< 0.001
		Seed	10.59 ± 2.42	4.25 ± 0.12	5.88 ± 0.38	< 0.001	< 0.001	< 0.002
	Ni	Bulb	ND	10.36 ± 1.29	5.86 ± 0.66	< 0.001	< 0.001	< 0.001
		Leaf	ND	13.22 ± 3.05	11.19 ± 1.41	< 0.001	< 0.001	= 0.251
		Seed	ND	18.77 ± 0.27	14.96 ± 0.23	< 0.001	< 0.001	< 0.001
	Zn	Bulb	15.16 ± 1.27	19.80 ± 2.19	17.17 ± 1.49	< 0.001	< 0.007	= 0.052
		Leaf	31.93 ± 1.05	32.40 ± 7.12	19.93 ± 1.38	= 0.251	< 0.001	< 0.001
		Seed	38.67 ± 1.48	28.35 ± 1.34	33.24 ± 1.50	< 0.001	< 0.001	< 0.001
% DM	Ca	Bulb	0.042 ± 0.01	0.037 ± 0.01	0.020 ± 0.01	< 0.001	< 0.001	< 0.001
		Leaf	1.72 ± 0.13	0.64 ± 0.02	0.58 ± 0.02	< 0.001	< 0.001	< 0.093
		Seed	0.13 ± 0.09	0.062 ± 0.01	0.026 ± 0.01	< 0.001	< 0.001	< 0.001
	Mg	Bulb	0.024 ± 0.01	0.053 ± 0.01	0.060 ± 0.02	< 0.001	< 0.001	< 0.001
		Leaf	0.16 ± 0.01	0.58 ± 0.03	0.61 ± 0.03	< 0.001	< 0.001	= 0.440
		Seed	0.069 ± 0.01	0.089 ± 0.01	0.11 ± 0.02	< 0.001	< 0.001	< 0.001

DM dry mass, *ND* not detected, *TL T. luanica*, *TK T. kosovarica*, *TA T. alabanica*

The data are presented by mean and standard error (±). Means in each row followed by same letters are not significantly different at *P* 0.05 and *P* 0.001

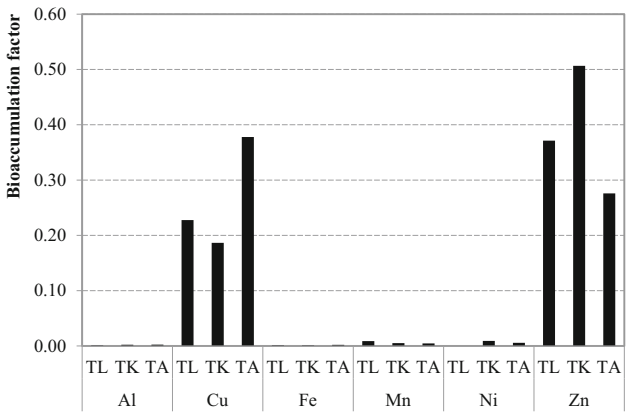

Fig. 1 Bioaccumulation factor (BF) of some metals (Al, Cu, Fe, Mn, Ni and Zn) in TL—*T. luanica*; TK—*T. kosovarica* and TA—*T. albanica*

leaves of these two tulipa species. Supporting this, Delfani et al. (2014) reported increased total chlorophyll content in leaves of black-eyed pea experimentally treated with Fe and Mg. It is known that low levels of some metals in soil had a beneficial effect on chlorophyll content (Stancheva et al. 2014).

The ALA content in leaves of *T. luanica* was significantly lower (15.02 μM g^{-1} FW) compared with *T. kosovarica* and *T. albanica* plants (19.16 and 23.72 μM g^{-1} FW, respectively).

There was an association between inhibition of ALA-D activity and increase of ALA content in leaves of *T. kosovarica* and *T. albanica.* The ALA-D enzyme catalyzes the asymmetric condensation of two molecules of ALA to porphobilinogen, and this process results in decrease of ALA content.

MDA and GSH contents were significantly higher in leaves of *T. luanica* compared with *T. kosovarica*. The increased content of MDA and GSH indicates the prevalence of oxidative stress, and this may be one of the possible mechanisms by which toxicity due to metal stress could be manifested in plant tissue. The higher amounts of MDA and GSH may be a consequence of higher

Table 4 δ-Aminolevulinic acid dehydratase (ALA-D) activity, δ-aminolevulinic acid (ALA), Total Chlorophyll, Malondialdehyde (MDA) and Glutathione (GSH) contents in leaves of *T. luanica, T. kosovarica* and *T. albanica*

Species		ALA-D (μM PGB mg prot.$^{-1}$ h^{-1})	ALA (μM g^{-1} FW)	Total Chl. (mg g^{-1} FW)	MDA (μM g^{-1} FW)	GSH (μM g^{-1} FW)
T. luanica		13.76 ± 3.42	15.02 ± 2.93	8.33 ± 0.78	1.97 ± 0.49	4.50 ± 0.72
T. kosovarica		10.98 ± 3.72	19.16 ± 3.22	8.86 ± 1.26	1.41 ± 0.49	3.30 ± 1.10
T. albanica		6.06 ± 2.46	23.72 ± 3.13	9.09 ± 1.87	1.80 ± 0.85	4.34 ± 1.72
Significance	TL:TK	= 0.100	< 0.001	= 0.276	< 0.020	< 0.010
	TL:TA	< 0.001	< 0.002	= 0.252	= 0.850	= 0.784
	TK:TA	< 0.003	< 0.002	= 0.749	= 0.230	= 0.125

FW fresh weight, *ALA-D* Delta-aminolevulinic acid dehydratase, *ALA* aminolevulinic acid, *Total chl.* total chlorophylls, *MDA Malondialdehyde*, *GSH* Gluthatione, *PGB* Porphobilinogen, *TL T. luanica*, *TK T. kosovarica*, *TA T. alabanica*

The data are presented by mean and standard error (±). Means in each column followed by same letters are not significantly different at *P* 0.05 and *P* 0.001

Table 5 Correlation between trace elements and δ-aminolevulinic acid dehydratase (ALA-D) activity in leaves of *T. luanica, T. kosovarica* and *T. albanica*

	Species	Al	Ca	Cu	Fe	Mg	Mn	Ni	Zn
ALA-D	*T. luanica*	0.786*	0.851**	0.840**	0.794**	0.798**	0.443	/	− 0.706*
	T. kosovarica	− 0.232	0.161	− 0.437	− 0.440	0.002	− 0.530	− 0.147	− 0.491
	T. albanica	0.130	0.718*	− 0.275	− 0.372	0.339	− 0.462	− 0.389	0.320

*$P < 0.05$; **$P < 0.001$

concentrations of Cu and lower concentrations of Mg in leaves of *T. luanica* plants. This result is in agreement with Panda (2008), who reported that higher concentrations of Cu in *Lemna minor* plants increased MDA and GSH levels. Mg deficiency conditions applied to spinach culture caused an oxidative stress proved by increase in GSH and MDA levels and antioxidant enzymes (Ze et al. 2009). However, our results of higher amounts of GSH in leaves of *T. luanica* are linked with higher synthesis in order to protect enzyme thiol groups from ions of metals. Glutathione is a peptide and plays a significant role in the redox balance of cells (Hasanuzzaman et al. 2017) and may act as a scavenger of ROS, an electron source for ascorbate regeneration, and a metal binding peptide. Stancheva et al. (2010), during their investigation with *Salvia officinalis,* have reported that high levels of heavy metals contained in soil are efficient generators of toxic oxygen species, which can initiate lipid peroxidation and increase levels of MDA and GSH as a result. According to Aydogan et al. (2017), Cu bioaccumulation in two bryophyte species induced an increase of lipid peroxidation levels (MDA) up to 155%.

Conclusions

Based on the higher concentration of some metals in soil and the level of accumulation or bioaccumulation factor (less than 1) in tulipa plants in native habitats, it is concluded that these plants are not hyperaccumulators. With respect to other metals, the leaves of *T. kosovarica* and *T. albanica* (serpentine sites) accumulated considerable quantities of Ni, Al and Fe in comparison with *T. luanica* (limestone site). In conclusion, the results of the present study suggest that ALA-D activity and contents of ALA, MDA and GSH are good bioindicators for evaluation of effects of metals in tulip native species from serpentine and limestone sites. It is suggested that ALA-D activity could be used as a sensitive biomarker for evaluation of oxidative stress in genus Tulipa plants growing in different native habitats.

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