10th INTERNATIONAL SCIENTIFIC CONFERENCE
RURAL DEVELOPMENT 2021
CHALLENGES FOR SUSTAINABLE BIOECONOMY AND CLIMATE CHANGE
21-23 September, 2021, Vytautas Magnus University Agriculture Academy, Lithuania

VYTAUTAS MAGNUS UNIVERSITY AGRICULTURE ACADEMY

Proceedings of the 10th International Scientific Conference Rural Development 2021
Edited by assoc. prof. dr. Judita Černiauskienė

ISSN 1822-3230 (Print)
ISSN 2345-0916 (Online)

Article DOI: http://doi.org/10.15544/RD.2021.038

HEAVY METAL TOLERANCE AND ACCUMULATION POTENTIAL OF COASTAL ACCESSIONS OF *TRIFOLIUM FRAGIFERUM*, A PROMISING FORAGE SPECIES

Gederts IEVINSH, Faculty of Biology, University of Latvia, 1 Jelgavas Str., Riga, Latvia; gederts.ievins@lu.lv *(corresponding author)*
Andis KARLSONS, Institute of Biology, University of Latvia, 4 O. Vaciesa Str., Riga, Latvia; andis.karlsons@lu.lv
Astra JĒKABSONE, Faculty of Biology, University of Latvia, 1 Jelgavas Str., Riga, Latvia; astra.jekabsone@lu.lv
Una ANDERSONE-OZOLA, Faculty of Biology, University of Latvia, 1 Jelgavas Str., Riga, Latvia; una.andersone-ozola@lu.lv

The aim of the present study was to examine heavy metal tolerance of different wild accessions of strawberry clover, *T. fragiferum*, a promising forage species, in comparison to a commercial cultivar, and to find out if there is a tendency to accumulate heavy metals in above ground parts of strawberry clover plants. Seeds from four geographically isolated wild populations of *T. fragiferum* in Latvia as well as cv. 'Palestine' were used to establish experiment in controlled conditions using substrate gradient of Cd and Pb. Similar to closely related species *T. repens, T. fragiferum* showed high tolerance to heavy metals Cd and Pb and excluded heavy metals from above ground parts. Some physiological differences were evident in respect to morphological responses of different accessions to the two heavy metals as well as regarding heavy metal accumulation potential in different plant parts. It was concluded that when cultivated in unpolluted soils, shoots of *T. fragiferum* can be considered safe as forage for animal consumption.

Keywords: crop wild relatives, heavy metals, strawberry clower

INTRODUCTION

Due to the need to ensure crop productivity in conditions of growing anthropogenic impact and global climate change, crop wild relatives (CWR) represent a valuable genetic source of tolerance-related characteristics (Zhang et al. 2016). CWRs can be used for development of new crop cultivars with better adaptation to growing environmental heterogeneity (Dempewolf et al. 2014).

In the Baltic Region, perennial forage legumes and grasses represent the main type of CWRs, with legumes being especially important for development of sustainable agricultural systems. Among them, different wild *Trifolium* species has been studied recently in the Baltic countries as pontential forage crops (Dabkevičienė, Dabkevičius 2005; Bērziņa et al. 2008; Paplauskienė, Dabkevičienė 2012). However, extremely rare wild clover species, *Trifolium fragiferum* L., has not been studied and practically used in Europe, but has been cultivated in other regions because of relatively good tolerance to soil salinity, alkalinity and flooding (Townsend 1985). In the Baltic Region, *T. fragiferum* exclusively occurs in an endangered habitat 'Baltic coastal meadow' (A2.5b; Janssen, Rodwell 2016).

The question of possible heavy metal accumulation in plant aboveground parts has an immense practical importance for forage species as it is desired that any heavy metal contaminants are accumulated in roots but excluded from shoots. Among *Trifolium* species, only two of them have been relatively intensively studied in respect to heavy metal tolerance and accumulation, namely, *Trifolium repens* (e.g., Lopareva-Pohu et al. 2011; Lambrechts et al. 2014; Lanier et al. 2016; Xiao et al. 2020; Lin et al. 2021) and *Trifolium alexandrinum* (e.g., Ali et al. 2012; Sinegani et al. 2015; Bhatti et al. 2018). There is no information available in the literature on heavy metal tolerance of *T. fragiferum*, but closely related *T. repens* has been characterized as heavy metal-tolerant excluder species (Lanier et al. 2016). In contrast, *T. alexandrinum* accumulated similar concentration of particular metals, including Cd and Pb, in both roots and shoots (Bhatti et al. 2018), and even has been suggested as species with high phytoextraction potential (Ali et al. 2012).

Recently we characterized abiotic stress tolerance of several wild accessions of *T. fragiferum* from coastal habitats of the Baltic Sea (Andersone-Ozola et al. 2021). Most importantly, it was shown that all accessions had relatively good tolerance against high soil moisture, trampling and cutting, but each accession from geographically isolated micropopulation had unique physiological profile. It seems to be highly likely that responses to other factors, including heavy metals, may significantly differ for different accessions of *T. fragiferum*. Therefore, the aim of the present study was to examine heavy metal tolerance of different wild accessions of *T. fragiferum* in comparison to a commercial cultivar, and to find out if there is a tendency to accumulate heavy metals in above ground parts of strawberry clover plants.

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MATERIALS AND METHODS

Seeds from four geographically isolated wild accessions of *T. fragiferum* in Latvia were used for propagation of plants used in the present study: TF1 (wet saline meadow, Liepāja), TF2 (saline river bank near estuary (Jūrmala, Lielupe), TF4 (degraded land in urban industrial area, Rīga, Skanste), TF7 (dry coastal meadow, Ainaži). *T. fragiferum* cv. 'Palestine' seeds were purchased from Sheffield's Seeds Company (USA) and used as a reference genotype (TF8). Experimental details on establishment of plant material and cultivation conditions are described elsewhere (Andersone-Ozola et al. 2021). Briefly, seeds were surface sterilized with 5% NaOCl, imbibed in water and scarified with scalpel. Prepared seeds were germinated in autoclaved substrate (Garden Soil, Biolan, Finland) in plastic plant tissue culture containers in a growth cabinet. Established seedlings with the two true leaves were individually transplanted first to 250 mL plastic containers and after two weeks to 1.3 L plastic containers filled with a mixture of heat-treated substrate containing Garden Soil and quartz sand (1:3, v/v). Plants were cultivated in an experimental automated greenhouse with supplemented light (380 µmol m^{-2} s^{-1} at the plant level) with 16 h photoperiod, day/night temperature 24/16 °C, relative air humidity 60 to 70%. Substrate water content was kept at 50 to 60% using deionized water. Plants were fertilized every other week with Yara Tera Kristalon Red and Calcinit fertilizers (Yara, Norway).

One week after the last transplantation the plants were randomly distributed in seven treatments, five individual plants per treatment: control, Cd 10, Cd 50, Cd 100, Pb 100, Pb 200, Pb 500 (in mg of metal per 1 L of substrate). For respective treatments, $CdCl_2$ and $Pb(CH_3COO)_2$ $3H_2O$ were used in necessary dilutions in deionized water. Plants were cultivated for four weeks after the treatment. At the termination, plants were individually separated in different parts (roots, stolons, flower stalks, inflorescences, leaf petioles, leaf blades) and both fresh and dry mass (after drying in an oven) were measured. Water content was calculated as g H_2O per g dry mass. As indicated previosuly (Andersone-Ozola et al. 2021), flower-related characteristics of *T. fragiferum* were extremely variable between individual plants, therefore, these parameters were further used only for calculation of total shoot biomass per plant, but were not analyzed as individual parameters.

Concentration of Cd and Pb was measured in dried material for all plant parts. For each plant sample approximately 2 g plant material was collected. Samples were fixed 2 to 3 min at 105 °C, then dried at 60 °C to constant weight and ground. Plant tissue test solution was prepared by dry ashing with HNO_3 vapor and re-dissolving in a 3% HCl solution (Rinkis et al. 1987). The testing solution was used for the determination of analyzed heavy metals. Microwave plasma atomic emission spectrometry (4200 MP-AES, Agilent) was used for the measurement of Pb and Cd according to manufacturer's instructions. Analyzed element concentrations in plant tissue were expressed as mg kg^{-1}.

Results were analyzed and graphs were made by KaleidaGraph (v. 4.1, Synergy Software, USA). Statistical significance of differences between all treatments was evaluated by one-way anova using Microsoft Excel spreadsheet (www.biostathandbook.com/anova.xls) (McDonald 2014).

RESULTS

Total dry mass of *T. fragiferum* shoots was relatively little affected by increasing concentration of heavy metals in susbtrate (Fig. 1). Only for Cd-treated plants, it significantly increased for TF7 at 10 mg kg^{-1} and decreased for TF8 at 100 mg kg^{-1}. Both root dry mass and leaf petiole dry mass were not significantly affected by any of treatments (data not shown). Dry mass of stolons was relatively more sensitive to heavy metal treatment. It significantly decreased for TF2 and TF4 at 50 mg L^{-1} Cd, and for all accessions at 100 mg L^{-1} Cd (Fig. 2A). However, in the case of Pb, treatment resulted in significant increase in dry mass of stolons for TF1 (by 11 and 25%) and TF8 (by 29 and 44%) at 100 and 200 mg L^{-1}, respectively (Fig. 2B).

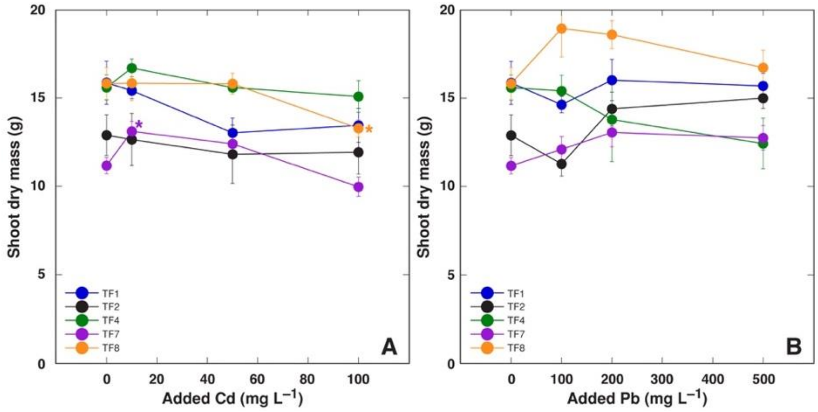

Figure 1. Effect of added substrate Cd (A) and Pb (B) on total shoot dry mass of different accessions of *Trifolium fragiferum*. Data are means ± SE from 5 replicates. Asterisks of respective color indicate statistically significant differences from control ($p < 0.05$).

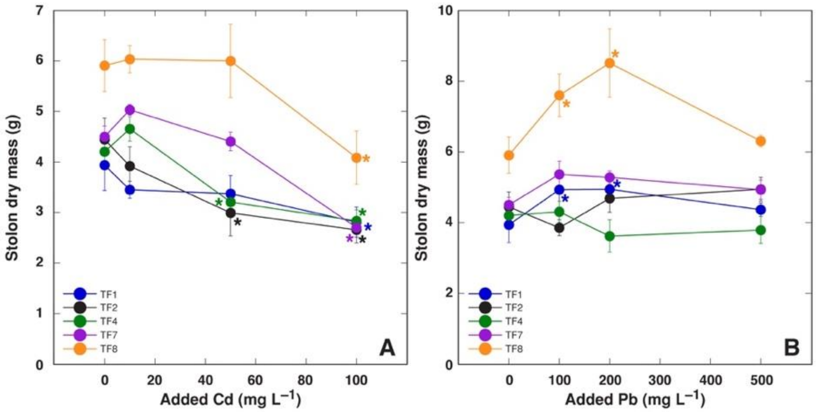

Figure 2. Effect of added substrate Cd (A) and Pb (B) on stolon dry mass of different accessions of *Trifolium fragiferum*. Data are means ± SE from 5 replicates. Asterisks of respective color indicate statistically significant differences from control ($p < 0.05$).

Leaf petiole dry mass was another parameter that was affected by heavy metal treatments: it significantly decreased for TF8 at 50 and for TF1, TF7 and TF8 for 100 mg kg^{-1} Cd (Fig. 3A). Besides, leaf petiole dry mass significantly decreased for TF7 for 100, 200, and 500 mg L^{-1} Pb (Fig. 3B). Water content in stolons, leaf petioles and leaf blades was not significantly affected by treatment with heavy metals (data not shown). However, root water content significanly decreased for TF1 by both Cd and Pb at all concentrations as well as for TF4 at 100 mg L^{-1} Cd (Fig. 4). To summarize, Cd tolerance decreased in an order TF7, TF1 > TF4 > TF2 > TF8, but Pb tolerance decreased in an order TF8 > TF1 > TF2, TF4 > TF7.

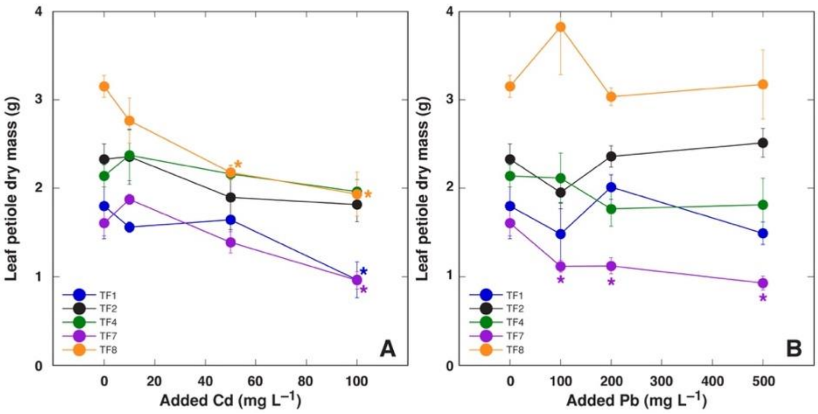

Figure 3. Effect of added substrate Cd (A) and Pb (B) on leaf petiole dry mass of different accessions of *Trifolium fragiferum*. Data are means ± SE from 5 replicates. Asterisks of respective color indicate statistically significant differences from control ($p < 0.05$).

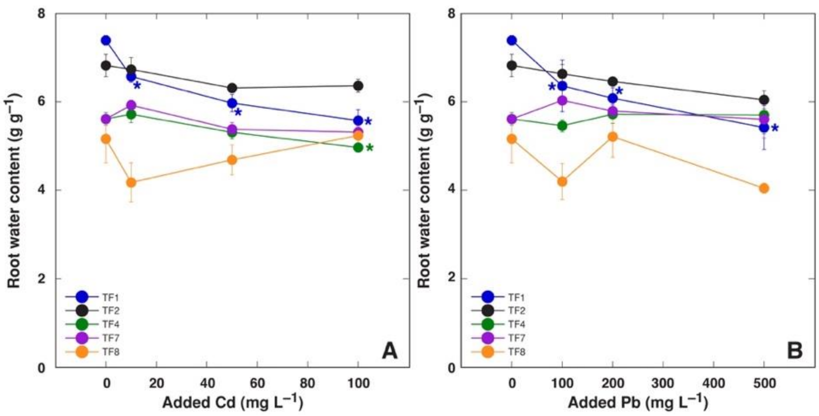

Figure 4. Effect of added substrate Cd (A) and Pb (B) on root water content of different accessions of *Trifolium fragiferum*. Data are means ± SE from 5 replicates. Asterisks of respective color indicate statistically significant differences from control ($p < 0.05$).

The accumulation potential of metals was markedly different in different parts of *T. fragiferum* plants. The lowest Cd concentration was in flower stalks (1.8 – 5.8 mg kg^{-1}) and flowers (3.3 – 7.5 mg kg^{-1}). The same relationship was seen for Pb, with its concentration in flower stalks reaching 2.3 – 9.4 mg kg^{-1} and that in flowers 2.5 – 8.1 mg kg^{-1}. Both Cd and Pb mainly accumulated in roots, with shoot concentrations being about 10 times lower for Cd (Fig. 5) and 5 to 20 times lower for Pb (Fig. 6) than these in roots.

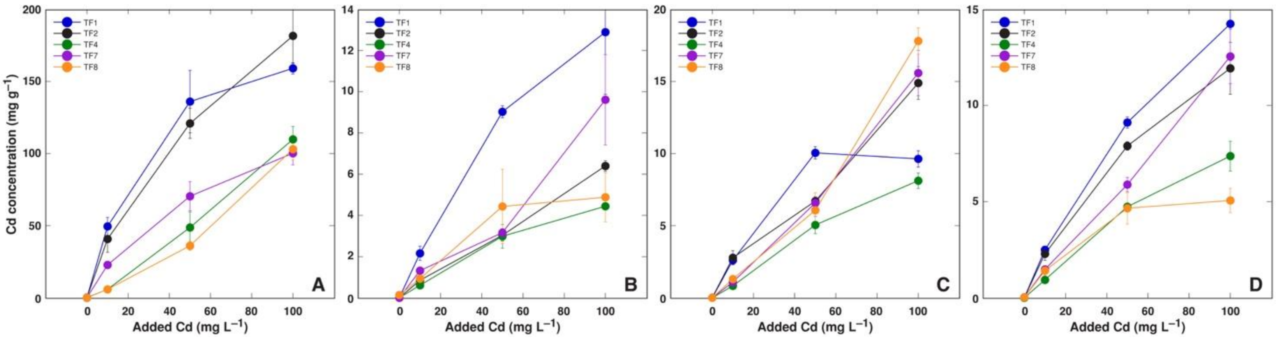

Figure 5. Cd concentration in roots (A), stolons (B), leaf petioles (C) and leaf blades (D) of different accessions of *Trifolium fragiferum* plants grown at different levels of substrate Cd concentration. Data are means ± SE from 3 replicates.

Differences in metal accumulation patterns were observed between different genotypes. Thus, accessions TF1 and TF2 had higher Cd accumulation capacity in roots in comparison to other accessions (Fig. 5A). Similarly, TF1 accumulated more Cd in stolons at all treatments (Fig. 5B). TF2, TF7 and TF8 accumulated more Cd in leaf petioles at high substrate Cd concentration in comparison to TF1 and TF4 (Fig. 5C); while TF1, TF2 and TF7 accumulated more Cd at moderate and high substrate concentrations in comparison to TF4 and TF8 (Fig. 5D). Accession-specific accumulation patterns were less pronounced for Pb, but in leaf blades saturation of accumulation was observed for TF4, TF7 and TF8 (Fig. 6D).

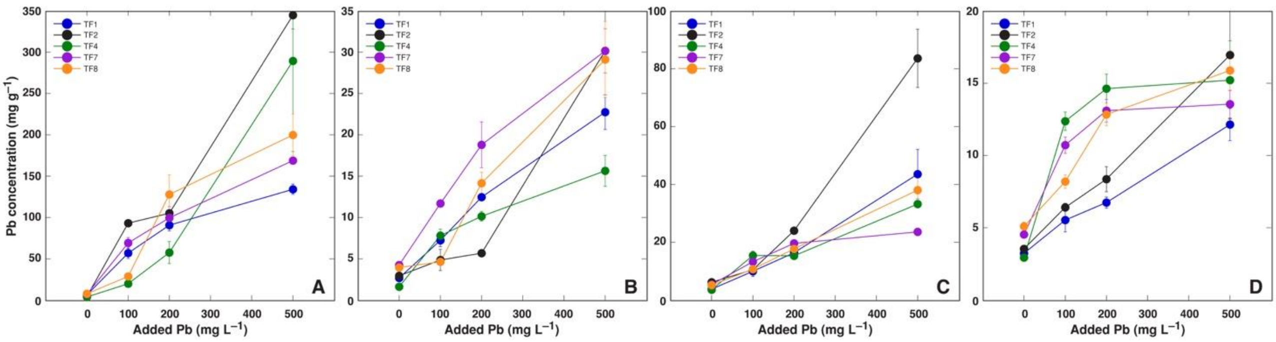

Figure 6. Pb concentration in roots (A), stolons (B), leaf petioles (C) and leaf blades (D) of different accessions of *Trifolium fragiferum* plants grown at different levels of substrate Pb concentration. Data are means ± SE from 3 replicates.

DISCUSSION

Tolerance to heavy metals and their accumulation ability in plant tissues are generally independent plant traits (Angulo-Bejarano et al. 2021). For example, even Cd hyperaccumulator species *Arabidopsis halleri* showed 45% decrease of shoot growth at 5 μM Cd, reaching 82% reduction at 100 μM (Zhao et al. 2006). In addition, tolerance to different heavy metals of particular species can vary significantly.

Visual symptoms of both Cd and Pb toxicity are usually associated with inhibition of plant growth and development (Pourrut et al. 2011). All accessions of *T. fragiferum* showed relatively good tolerance to increased substrate level of Cd and Pb as shown by absence of changes in root biomass and only extremely limited effect on total shoot biomass. However, several individual parts of shoot were significantly affected by heavy metal treatments although differently for different accessions. It is especially intriguing that the accession most tolerant to Cd (TF7) was the most sensitive to Pb, but the accession most tolerant to Pb (TF8) was the most sensitive to Cd. Decrease in tissue water content due to heavy metal treatment, as found in roots of TF1, relatively tolerant to both metals (Fig. 4), is considered to be the result of disruption of respiration and oxidative phosphorilation (Sharma, Dubey 2005).

Estimation of accumulation of heavy metals in above ground parts of *T. fragiferum* is important for its use as animal feed. Only one study can be found in the literature on heavy metal accumulation in *T. fragiferum* plants showing that heavy use of urban compost does not affect Cd (< 1 mg kg^{-1}) and Pb (8 mg kg^{-1}) concentration in leaves (Murillo et al. 1997). However, there were no experiments performed so far on effects on growth and metal accumulation at elevated soil concentration of heavy metals for *T. fragiferum*.

The global estimated range for Cd concentration in unpolluted soils is between 0.06 and 1.1 mg kg^{-1}, but average value for Pb is considered to be 25 mg kg^{-1}, with maximum concentration less than 100 mg kg^{-1} (Kabata-Pendias, Mukherjee 2007). At minimum Cd (10 mg L^{-1}) and Pb (100 mg L^{-1}) treatments used in the present study, maximum metal accumulation in above-ground parts was in leaf petioles, with mean values for all accessions 1.7 and 12 mg kg^{-1}, for Cd and Pb, respectively. With the average water content in leaf petioles being 4.5 g g^{-1} dry mass, respective metal concentrations on fresh mass basis were 0.3 mg kg^{-1} for Cd and 2.2 mg kg^{-1} for Pb. Consequently, even at the maximum level of Cd and Pb in relatively unpolluted agricultural soil, accumulation potential of Cd and Pb in above ground parts of *T. fragiferum* could be significantly lower than permissable maximum concentration of these metals in forage material (1 mg kg^{-1} fresh mass for Cd and 30 mg kg^{-1} for Pb) (EFSA 2004a; EFSA 2004b). There is no doubt that cultivation of

T. fragiferum in heavily metal-contaminated soils will leed to accumulation of these heavy metals in above ground parts well above permissable levels. However, both heavy metal tolerance and accumulation potential in natural conditions can be significantly affected by symbiosis with arbuscular mycorrhiza and associated rhizobacteria, as in the case of *T. repens* (Oleńska et al. 2020; Xiao et al. 2020; Xiao et al. 2021) and *T. pratense* (Vivas et al. 2003).

A pronounced physiological gradient of heavy metal concentration was evident for *T. fragiferum* plants in respect to both metals. Cd concentration decreased in an order roots > leaf petioles > leaf blades > stolons > flowers > flower stalks, but Pb concentration decreased in an order roots > leaf petioles > stolons > leaf blades > flowers > flower stalks. This is consistent with a general opinion that, for a majority of plants, Cd is preferentially accumulated in roots, with significantly lower concentration in leaves and even less in reproductive organs (Grant et al. 1998). Only Cd hyperaccumulator species can reach shoot Cd concentration similar to that in roots (Zhao et al. 2006) or even higher (Roosens et al. 2003). Similarly, Pb concentration in various plants decreases in an order roots > leaves > stems > inflorescences > seeds (Sharma, Dubey 2005). In contrast, as an extreme example, *Fagopyrum esculentum* plants accumulated as much as 8000 mg kg^{-1} Pb in leaves, with root concentration reaching 3300 mg kg^{-1} with no negative effect on plant growth (Tamura et al. 2005). Accumulation range for Cd in *T. fragiferum* was relatively lower to that of *T. repens*, where shoot Cd concentration reached 30 mg kg^{-1}, with root concentration being 500 mg kg^{-1} (Xiao et al. 2021). In respect to Pb, values very similar than these found in the present study were reported for *T. repens*: 167 mg kg^{-1} in roots and 36 mg kg^{-1} in shoots (Bidar et al. 2007).

Cd toxicity usually appears at tissue concentration of 5 – 10 mg kg^{-1}, but that for Pb at 10 – 20 mg kg^{-1} (White, Brown 2010). These concentrations were clearly exceeded in the present study. However, no direct relationship between tissue heavy metal concentration in various accessions of *T. fragiferum* and their tolerance has been observed. Similarly, even plants native to mine tailing area with potential for Cd phytostabilization, such as *Athyrium wardii*, showed significant reduction of shoot biomass at internal Cd concentration as low as 5 mg kg^{-1}, further decreasing by 62% at 50 mg kg^{-1} internal Cd (Zhang et al. 2012). The same species accumulated up to 4000 mg kg^{-1} Pb in shoots, with only 24% decrease in shoot biomass, possibly due to efficient compartmentalization of the metal in vacuoles of leaf cells (Zhao et al. 2015). For *T. fragiferum*, the most Cd tolerant accession TF7 accumulated 12 – 15 mg kg^{-1} Cd in leaves and 10 mg kg^{-1} Cd in stolons (Fig. 6) with 40% reduction in leaf petiole and stolon biomass (Fig. 2A). Accession TF8, being most tolerant to Pb, showed no negative effect on shoot growth while accumulating 29 mg kg^{-1} Pb in stolons and even 38 mg kg^{-1} Pb in leaf petioles (Fig. 7). Moreover, 44% stimulation of stolon growth for this accession was achieved (Fig. 2B) at internal Pb concentration in stolon tissues reaching 14 mg kg^{-1} Pb (Fig. 7). Thus, accessions of *T. fragiferum* can be characterized as heavy metal tolerant species excluding metals from above ground parts.

CONCLUSIONS

Several wild accessions of *T. fragiferum* from coastal habitats of the Baltic Sea show high tolerance to heavy metals Cd and Pb. However, some physiological differences are seen in respect to morphological responses of different accessions to the two heavy metals.

Similar to closely related species *T. repens, T. fragiferum* excludes heavy metals from above ground parts. Even at high substrate heavy metal level, their concentration in above ground parts is many times lower than that in roots. When cultivated in unpolluted soils, shoots of *T. fragiferum* can be considered safe as forage for animal consumption.

Acknowledgments. The study was supported by the Latvian Science Council project lzp-2020/2-0349 “Molecular, physiological and ecological evaluation of Latvian genetic resources of valuable wild legume species, *Trifolium fragiferum*, in a context of sustainable agriculture”.

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