Accumulation of Cd, Pb and Zn in *Tribulus terrestris* L. Grown on Industrially Polluted Soil and Plant Antioxidant Response

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ABSTRACT

Possibilities for phytoaccumulation of heavy metals and antioxidant capacity of puncture vine, grown on industrially polluted with Cd, Pb and Zn soil were studied. Content of Cd, Pb and Zn in the polluted soil exceeded permissible concentrations 3, 4 and 2 times respectively. Puncture vine plants (*Tribulus terrestris* L.), were grown under glasshouse conditions on polluted and non-polluted control soil. Plants grown on heavy metal polluted soil accumulated in the aboveground parts 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals concentration in plant and soil samples were determined on the inductively – coupled Plasma Mass Spectrometer. Spectrophotometric quantification of ascorbate, reduced glutathione and vitamin E was performed through the formation of phosphomolybdenum complex. Total antioxidant capacity (free radicals scavenging activity) was measured from the bleaching of the purple-colored methanol solution of free stable radical (diphenylpycril-hydrazyl, DPPH) inhibition. All antioxidant enzymes (ascorbate peroxidase, catalase, dehydroascorbate reductase, guaiacol peroxidase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and monodehydroascorbate reductase) were assayed spectrophotometrically. Puncture vine plants possess good ability to accumulate heavy metals. Plants grown on heavy metal polluted soil accumulated heavy metals in both the shoots and roots. Cd and Pb accumulated more in the roots than in the shoots of plants both from the non - polluted and polluted soil. The observed levels of main contaminates in aboveground parts were 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals content in the roots of treated plants was 2.5, 2.8 and 1.4 times more than in the controls for Cd, Pb and Zn respectively. The levels of heavy metals accumulation in aboveground parts allowed supposing that *Tribulus terrestris* is a plant that could be used for phytoremediation, more over that higher Cd and Zn levels were found in the plants than in the soil. Absence of biomass reduction indicated that puncture vine plants tolerate the existing concentration level of Cd, Pb and Zn. The antioxidant potential of the puncture vine plants is defined by the content of antioxidant metabolites vitamin E, ascorbate, glutathione and total phenols and antioxidant enzyme activities of glutathione peroxidase, glutathione reductase and dehydroascorbate reductase. From the results we can conclude that soil Cd, Pb and Zn in concentrations far exceeded permissible limit concentrations influenced only a part of antioxidant capacity of *Tribulus terrestris* plants.

**Key words:** *Tribulus terrestris* L. - heavy metals - antioxidants metabolites – antioxidant enzymes
Introduction

*Trifolium repens* is known for its use in the traditional medicine of many countries for the treatment of cancer and other diseases, edema, skin itch and wounds and impotence [11]. It is also included in many dietary supplements with biostimulating activity. *T. repens* is a rich source of furostanol and spirostanol saponins [31,32] and flavonoids [24]. Some medicinal plants are capable to accumulate heavy metals from contaminated soils [33]. Accumulation and hyper-accumulation of heavy metals is very important assumption for clearing up of contaminated substrates by plants – phytoremediation. Phytoremediation as a green biotechnology is a low-cost alternative to the traditional remediation technologies [25,26].

Toxic heavy metals in plant tissues are major problems that can affect both plant productivity and safety as food, herb and feed crops [2]. In regard to environmental biotechnologies, vascular plants developed different defense mechanisms against toxic metal stress. To combat the metal toxicity and produced several free radicals there is a mobilization of the antioxidant reserves in the plant that react both enzymatically and non-enzymatically resulted in neutralization of these toxic molecular species. There is an increasing interest in the use and measurement of antioxidants in the food, pharmaceutical and cosmetic industries. Non-enzymatic antioxidants include ascorbate (ASC) and glutathione (GSH) and enzymatic antioxidants are superoxide dismutase (SOD), different specific peroxidases, catalase (CAT) and enzymes of ascorbate-glutathione cycle as reviewed by Ahmad et al. [1]. It is well known that antioxidant defense system in plants is responsible for the detoxification of harmful reactive oxygen species (ROS).

The aim of this study was to evaluate the extent of accumulation of Cd, Pb and Zn in *Trifolium repens* and the effect of heavy metals uptake on the plant biomass and antioxidant capacity.

Materials and Methods

*Trifolium repens* L plants were grown starting from seeds in a climatic chamber at 12 h photoperiod, day/night temperature 25/18°C and photon flux density of 95 mmol m⁻² s⁻¹ until 21st day. Two-month old seedlings were transferred to 5 kg plastic pots (2 plants per pot) and were grown another 2 months until early fruit ripeness stage on the soil/sand substrate in the ratio 3:1.

Water was added to make up about 60% of water holding capacity. The soil was collected from the vicinities (1 km) of a Non-Ferrous Metals Combine with pH(H₂O) – 7.35 and the following content of heavy metals (µg g⁻¹DW): Cd - 9.02, Cu - 82.10, Pb - 301.75, Zn - 641.60. Because the Bulgarian permissible limit concentrations at pH(H₂O) – 7.35 are Cd - 3.0, Cu < 260, Pb < 80 and Zn < 340 µg g⁻¹DW the soils are heavily polluted with Cd, Pb and Zn. For the control non-polluted leached cinnamonic forest soil (Chromic Luvisols – FAO) was used (pH(H₂O) - 6.2) with the following content of studied heavy metals (µg g⁻¹DW): Cd – 0.125, Cu - 22.83, Pb - 16.00, Zn - 46.03.

Heavy metals accumulation:

soil samples were air dried and ground using a mortar and pestle, and then were sieved through a 0.149 mm sieve. The plant and soil samples were digested in a solution containing 3:1 (v/v) HNO₃:HClO₄ solution. The samples were heated on a heating block at 200°C to evaporate the samples to dryness. The residue was taken up in 25 ml of 1N HCl. Metal concentration were determined on the inductively – coupled Plasma Mass Spectrometer (CCD Simultaneus ICP OES, Varian, Austria).

Antioxidant enzyme analyses:

In order to prepare crude extracts for determination of enzymes of the ascorbate-glutathione cycle – glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR), with 4 cm³ of extraction buffer (100 mM KH₂PO₄, pH 7.8; 5 mM EDTA; 2% PVP (MW = 44,000) that was added to 0.3 g of tissue powder. The extraction buffer for the determination of dehydroascorbate reductase (DHAR) contained: 50 mM KH₂PO₄, pH 7.0; 1 mM ascorbate; 1 mM EDTA; 0.2% PVP and was added to 0.15 g of tissue powder. The suspensions were centrifuged (16,000 .serializer min, 4°C). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27°C using UV-VIS SPECORD or SPECOL 11. GPX (EC 1.11.1.9) according to Edwards [12], GST (EC 2.5.1.18) according to Li et al. [17], GR (EC 1.6.4.2) according to Sherwin and Farrant [27], MDHAR (EC 1.6.5.4.) according to Miyake and Asada [18], DHAR (EC 1.8.5.1.) according to Doulis et al. [9].

For determination of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPO) fresh shoot material (0.250 g FW) was homogenized in 0.1 M K-phosphate buffer, pH 7.0 containing 1.0 mM Na₂-EDTA and 1% (w/v) polyvinylpirrolidone. Because APX is inactivated in the absence of ascorbate, 5.0 mM ascorbic acid was supplemented to the extraction buffer. The homogenate was centrifuged at 14,000 g for 30 min and the supernatant was immediately used...
as a crude enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0–4°C. Enzyme activities were determined spectrophotometrically at 25°C according to the following protocols: SOD [EC 1.15.1.1] [4], CAT [EC 1.11.1.6] [5], APX [EC 1.11.1.11] [19] and GPO [EC 1.11.1.7] [8]. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of riboflavin-mediated NBT (nitroblue tetrazolium) reduction. Soluble protein content was determined by the method of Bradford [6] using bovine serum albumin as a standard.

Other antioxidants:

Spectrophotometric quantification of ascorbate (ASC), reduced glutathione (GSH) and vitamin E was performed through the formation of phosphomolybdenum complex. The assay was based on the reduction of Mo(VI) to Mo(V) by the sample analysis and the subsequent formation of a phosphate-Mo(V) at acidic pH [22]. The method has been optimized and characterized with respect to linearity interval, repetitively and reproducibility, and molar absorption coefficients for the quantitation of ascorbate, glutathione and vitamin E. Absorption coefficients were: (3.4±0.1) x 10^3 M^{-1} cm^{-1} for ascorbic acid, (2.7 ±0.2) x 103 M^{-1} cm^{-1} for glutathione and (4.0±0.1) x 10^3 M^{-1} cm^{-1} for α-tocopherol.1 Total antioxidant capacity (free radicals scavenging activity) was measured from the bleaching of the purple-colored methanol solution of free stable radical (diphenylpyrrol-hydrazyl, DPPH•) inhibition after Tepe et al. [29]. DPPH• radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The inhibition of free radical DPPH• in percent (I%) was calculated in the following way: I% = (A_{blank}-A_{sample}/A_{blank}) x 100, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, i.e. puncture vine extracts.

The level of lipid peroxidation as 2-thiobarbituric acid reactive metabolites, chiefly malondialdehyde (MDA) and H_{2}O_{2} content were determined as described previously (Heath and Packer, 1968).

For determination of the phenols and flavonoids fresh leaves samples (1 g) were ground and exhaustively extracted with 96% (v/v) methanol. Content of phenolic compounds were determined spectrophotometrically using Folin-Ciocalteu reagent and calculated as caffeic acid equivalents [21]. Flavonoids in plant tissues were measured by Zhishen et al. [30] spectrophotometrically using standard curve of catechin.

Data are expressed as means ±SE, where n = 3. Comparison of means was performed by Fisher’s LSD test (P ≤ 0.05) after performing ANOVA analysis (Statgraphics Plus, v. 2.1).

Results and Discussion

The content of heavy metals in soil decreased after plant harvest in comparison with their initial levels (Table 1). Puncture vine plants possess good ability to accumulate heavy metals. In case of plant growth on heavy metal polluted soil higher levels of main contaminates in aboveground parts were observed – 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals content in the roots of treated plants was 2.5, 2.8 and 1.4 times more than in the controls for Cd, Pb and Zn respectively. Metal accumulator plant species can concentrate metal in their aerial parts, to levels far exceeding than soil. The levels of heavy metals accumulation in aboveground parts allowed supposing that Tribulus terrestris is a plant that could be used for phytoremediation, more over that higher Cd and Zn levels were found in the plants than in the soil. Cd and Pb were accumulated more in the roots than in the shoots of plants both from the non-polluted and polluted soil. In the polluted plants, 2.3 times more Cd and 2.8 times more Pb were observed in the roots than in the shoots. High mobility of Zn resulted in higher accumulation of this essential metal previously in the shoots of polluted plants (Table 1). A comparative study of heavy metals distribution in Tribulus terrestris of Hussain et al. [15] showed that the concentration of Zn is in the order stem>root>seeds>leaves which is in correspondence with our results. On the other hand the authors showed that the order for Pb is seeds>stems>leaves>roots, which is not supported by our results.

Hyperaccumulators are plants that can absorb high levels of contaminants concentrated either in their roots, shoots and/or leaves [23,7,3] have defined metal hyperaccumulator as plants that contain more than or up to 0.1% of copper, cadmium, chromium, lead, nickel cobalt or 1% of zinc or manganese in the dry matter. For cadmium and other rare metals, it is > 0.01% by dry weight.

Heavy metal pollution with Cd, Zn and Pb did not result in shoots, seeds and roots fresh and dry biomass inhibition (Table 2). Moreover, fresh weight of shoots, seeds and roots dry weight of shoots and roots of treated plants was higher than this for the control plants. Only seeds dry weight did not significantly differ between the treatments.

Therefore, absence of biomass reduction indicated that puncture vine plants tolerate the existing concentration level of Cd, Pb and Zn.
Table 1: Content of heavy metals in control (C) and polluted (P) soils before planting and after harvesting of *Tribulus terrestris* and metals accumulation in the roots and shoots.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cd</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>µg g⁻¹ DW</td>
<td>µg g⁻¹ DW</td>
<td>µg g⁻¹ DW</td>
</tr>
<tr>
<td>Before planting (C)</td>
<td>0.125±0.006°</td>
<td>16.000±0.800°</td>
<td>46.025±2.301°</td>
</tr>
<tr>
<td>After harvesting (C)</td>
<td>0.88±0.004*</td>
<td>9.000±0.525*</td>
<td>37.320±1.528*</td>
</tr>
<tr>
<td>LSD</td>
<td>0.012</td>
<td>1.538</td>
<td>4.428</td>
</tr>
<tr>
<td>Before planting (P)</td>
<td>9.020±0.451°</td>
<td>301.750±15.088°</td>
<td>641.600±32.080°</td>
</tr>
<tr>
<td>After harvesting (P)</td>
<td>7.700±0.384*</td>
<td>219.500±11.225*</td>
<td>509.850±28.492*</td>
</tr>
<tr>
<td>LSD</td>
<td>0.949</td>
<td>30.152</td>
<td>68.777</td>
</tr>
<tr>
<td>Plants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control plants - shoots</td>
<td>3.240±0.16°</td>
<td>38.240±0.82a</td>
<td>262.390±10.180a</td>
</tr>
<tr>
<td>Treated plants - shoots</td>
<td>10.770±0.54°</td>
<td>163.190±4.420b</td>
<td>613.820±20.038b</td>
</tr>
<tr>
<td>LSD</td>
<td>0.903</td>
<td>7.106</td>
<td>35.966</td>
</tr>
<tr>
<td>Times of increase*</td>
<td>3.3</td>
<td>4.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Control plants - roots</td>
<td>9.630±0.26°</td>
<td>163.970±8.198a</td>
<td>300.070±15.048a</td>
</tr>
<tr>
<td>Treated plants - roots</td>
<td>24.400±0.782b</td>
<td>463.000±23.080b</td>
<td>427.300±19.438b</td>
</tr>
<tr>
<td>LSD</td>
<td>1.318</td>
<td>34.529</td>
<td>39.310</td>
</tr>
<tr>
<td>Times of increase</td>
<td>2.5</td>
<td>2.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Times of increase toward the control

Values are means ± SE, n=6; different letters indicate significant differences assessed by Fisher LSD test (P<0.05) after performing ANOVA analysis.

Table 2: Fresh and dry weight of puncture vine organs affected by the high heavy metals levels

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fresh weight g plant⁻¹</th>
<th>Dry weight g plant⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control plants</td>
<td>Shoots 2.063±0.103</td>
<td>Shoots 2.035±0.025</td>
</tr>
<tr>
<td></td>
<td>Seeds 2.753±0.135</td>
<td>Seeds 2.675±0.046</td>
</tr>
<tr>
<td></td>
<td>Roots 0.265±0.013</td>
<td>Roots 0.099±0.005</td>
</tr>
<tr>
<td>Treated plants</td>
<td>Shoots 2.782±0.136</td>
<td>Shoots 3.751±0.037</td>
</tr>
<tr>
<td></td>
<td>Seeds 3.674±0.183</td>
<td>Seeds 3.957±0.047</td>
</tr>
<tr>
<td></td>
<td>Roots 0.421±0.021</td>
<td>Roots 0.171±0.008</td>
</tr>
</tbody>
</table>

Values are means ± SE, n=6; different letters indicate significant differences assessed by Fisher LSD test (P<0.05) after performing ANOVA multifactor analysis.

The level of antioxidant metabolites (Figure 1) and enzymes (Figure 2) indicates the antioxidant potential of puncture vine plants. Antioxidant metabolites such as ascorbate, glutathione, total phenols and especially vitamin E increased in the seeds of puncture vine plants, but total antioxidant activity decreased along with other antioxidants such as H₂O₂, MDA and flavonoids (Figure 1). A stable DPPH radical was used to investigate scavenging activity of puncture vine extracts. Toxic O₂ species can initiate lipid peroxidation and increased levels of MDA as a result. Lowered levels of DPPH; MDA and H₂O₂ in plants grown on heavy metals polluted soil led to assumption that oxidative stress in plant tissues caused by heavy metal pollution was not strongly expressed.

Among the low-molecular-weight antioxidants, ASC and GSH fulfill multiple physiological roles in defense reactions. Glutathione is a precursor of the phytochelatins, which are responsible for controlling cellular heavy metal concentration [13], therefore increased GSH levels are connected with enhanced plant tolerance to stress. Total phenols and flavonoids are also involved in plant antioxidant defense. It has already been reported that the antioxidant mechanism of flavonoids may also come from the interaction between transition-metal ions and flavonoids to produce complexes that keep the metal ions from their participation in free-radical generation [30]. H₂O₂ can also be produced by a number of non-enzymatic and enzymatic processes in cells while mitochondria and chloroplasts are the major sources of H₂O₂ in the cells, peroxisomes and glyoxysomes also contain SOD and APX, which are responsible for its production and scavenging [16].

Despite the reduced H₂O₂ content, CAT activity slightly increased in plants grown on heavy metals polluted soil (Figure 2). Among the enzymes of ascorbate-glutathione cycle, activity of GPX, GR and DHAR also increased in comparison with the controls. Two other peroxidases, GPO and APX potential scavengers of H₂O₂ decreased due to the heavy metal pollution in correspondence with the lowered hydrogen peroxide content. Elevated activity of DHAR and lessening of APX coincided with higher antioxidant content in polluted plant tissues (Figure 1-2). On the other hand, little increase of reduced glutathione in polluted plants (Figure 1) could be due to high GPX activity, compensated with elevated GR. SOD that catalyze the dismutation of superoxide into O₂ and H₂O₂ also decreased due to the presence of heavy metals (Figure 2). Therefore, antioxidant defense of *Tribulus terrestris* grown on heavy metals polluted soil to reactive oxygen species was not expressed clearly as GPO, SOD, APX and GST indicated. GSTs are considered, among several
Fig. 1: Total antioxidant potential (DPPH) and contents of hydrogen peroxide (H$_2$O$_2$), reduced (GSH) glutathione, vitamin E, ascorbate (ASC) and malondialdehyde (MDA), phenols and flavonoids in the shoots of *Tribulus terrestris* L., grown on non-polluted control soil (C) and heavy metals polluted soil (HM). Values are means ± SE, n=3; different letters indicate significant differences assessed by Fisher LSD test (P<0.05) after performing ANOVA multifactor analysis.

Fig. 2: Activity of catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR), guaiacol peroxidase (GPO), superoxide dismutase (SOD), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) in the shoots of *Tribulus terrestris* L., grown on non-polluted control soil (C) and heavy metals polluted soil (HM). Values are means ± SE, n=3; different letters indicate significant differences assessed by Fisher LSD test (P<0.05) after performing ANOVA multifactor analysis.
others, to contribute to the biotransformation of xenobiotics [10]. Reactive oxygen species are generated in the plant cells under normal metabolism and predominantly under stress conditions, but ROS such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ are synthesized at high rates even under optimal conditions [20]. These antioxidant systems can be divided into two categories: one that reacts with ROS and keeps them at low levels, (peroxidases, SOD and CAT), and one that regenerates the oxidized antioxidants (APX and GR) [28].

**Conclusion:**

Puncture vine plants possess good ability to accumulate heavy metals. Plants grown on heavy metal polluted soil accumulated heavy metals in both the shoots and roots. Cd and Pb accumulated more in the roots than in the shoots of plants both from the non-polluted and polluted soil. The observed levels of main contaminants in aboveground parts were 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals content in the roots of treated plants was 2.5, 2.8 and 1.4 times more than in the controls for Cd, Pb and Zn respectively. The levels of heavy metals accumulation in aboveground parts allowed supposing that *Tribulus terrestris* is a plant that could be used for phytoremediation, more over that higher Cd and Zn levels were found in the plants than in the soil. Absence of biomass reduction indicated that puncture vine plants tolerate the existing concentration level of Cd, Pb and Zn. The antioxidant potential of puncture vine plants is determined by the content of antioxidant metabolites vitamin E, ascorbate, glutathione and total phenols and antioxidant enzyme activities of GPX, GR and DHAR. From the results, we can conclude that soil Cd, Pb and Zn in concentrations far exceeded permissible limit concentrations influenced only a part antioxidant capacity of *Tribulus terrestris* plants.

**Abbreviations:**

APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHAR, dehydroascorbate reductase; DHASC, dehydroascorbate; GPO, guaiacol peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; MDHAR, monodehydroascorbate reductase; MDA, malondialdehyde; ROS, reactive oxygen species

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