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ANTIOXIDANT RESPONSES TO ELEVATED LEVELS OF Cd AND Pb IN *TRIBULUS TERRESTRIS* PLANTS

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Summary: Puncture vine (*Tribulus terrestris* L.) plants grown on non-polluted and polluted with Cd and Pb soils were analyzed with reference to the heavy metal effects on antioxidant metabolites and enzymes as well as boiactive compounds in the above-ground plant parts. Plants responded to the exposure to heavy metals with a decrease in the levels of malondialdehyde, hydrogene peroxide (in shoots), ascorbate, glutathione and lipid soluble antioxidants, expressed as α-tocopherol (in shoots), and an increase in the content of total phenols in fruits. The total antioxidant activity was stimulated only in shoots of stressed plants. Antioxidant defense against heavy metal accumulation was based mainly on the increased activities of catalase and three peroxidases (guaiacol peroxidase, glutathione peroxidase, and ascorbate peroxidase) in fruits and shoots of *Tribulus terrestris*. Catalase and peroxidases were found to act together in scavenging hydrogen peroxide. The functional activity of the ascorbate-glutathione cycle in plants grown under the experimental conditions was not effective enough. Despite the routine concentration decreased due to heavy metal pollution, the content of furostanol saponins protodioscin and prototribestin was significantly higher.

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Abbreviations: AGC – ascorbate glutathione cycle; APX – ascorbate peroxidase; ASC – ascorbate; CAT – catalase; DHAR – dehydroascorbate reductase; DHASC – dehydroascorbate; GPO – guaiacol peroxidase; GPX – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; GST – glutathione-S-transferase; MDHAR – monodehydroascorbate reductase; MDA – malondialdehyde; MP – medicinal plant; PLC – permissible limit concentrations; ROS – reactive oxygen species.

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INTRODUCTION

Heavy metal (HM) contamination of agricultural soils is a major environmental problem that can reduce both the productivity of plants and the safety of plant products as foods and feeds (Alloway, 1990). The success of phytoremediation depends on plant growth rate and capacity to accumulate high metal concentrations in plant shoots. Many plant species, identified as hyperaccumulators, are not suitable for phytoremediation application in the field, because they grow slowly and accumulate small biomass (Baker and Books, 1989). Some medicinal plants (MP) however, can be cultivated as alternative crops in heavy metal polluted agricultural soils due to their significant phytoextraction potential and the fact that the metal content in their essential oils is negligible (Zheljazkov et al., 2006). *T. terrestris* L. (Zygophyllaceae) is an annual herb which is widely used in treatment of sexual disorders (Brown et al., 2001), but it has also shown antirheumatic, analgesic, diuretic and uricosuric effects (Akram et al., 2011). The tribulus extract contains saponins (Wang et al., 1997), flavonoids (Saleh et al., 1982), amides and alkaloids (Wang et al., 1997) which are responsible for its biological activities. The plants from Bulgaria are wealthy of two dominating furastanol saponins – protodioscin and prototribestin (Kostova and Donchev, 2005) and represent a source for the industrial production of the medicine, which supplements the increase of the sex drive of men and women. Stancheva et al. (2011) reported that *T. terrestris* plants grown on industrially polluted soil under conditions of pot experiment showed good ability for Cd, Pb and Zn accumulation.

All HMs are toxic for plants at high concentrations and one of the reasons for this effect is that they may cause oxidative stress by generating free radicals and reactive oxygen species (ROS) in plant tissues (Sharma et al., 2010). These species react with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage and thus affect cell viability. The antioxidative system of plants comprises several enzymes: superoxide dismutase (SOD), peroxidases, catalases (CAT), enzymes of ascorbate-glutathione cycle (AGC) and low molecular mass nonenzymatic antioxidant metabolites such as ascorbic acid (ASC), glutathione (GSH), tocopherols (Sharma et al., 2010). Superoxide radicals generated in plant cells are converted to H_2O_2 by the action of SOD. The accumulation of H_2O_2, a strong oxidant, is prevented in the cell either by CAT, peroxidases or by AGC, where ascorbate peroxidase (APX) reduces it to H_2O. Monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) involved in AGC play regulatory roles in the HM-induced oxidative stress and their increased activity has been presumed to be a metal detoxification response (Noctor and Foyer, 1998).

The present paper aimed at establishing the possible antioxidative mechanisms that could be operational in leaves and fruits of *T. terrestris* grown under field conditions on industrially polluted with Cd and Pb soil. The effects of HMs on plant productivity and quantity of steroidal saponins were studied in the context of the possibility to use this MP as an alternative crop for cultivation in polluted agricultural soils.

MATERIALS AND METHODS

Tribulus terrestris L. plants were grown under field conditions on two (control and polluted with Cd and Pb) experimental fields. The control field with non-polluted leached cinnamonic forest soil (Chromic Luvisols – FAO) possessed the following agrochemical characteristics: pH(H_2O) = 6.2, soil total mineral nitrogen (N-NO_3^- + N-NH_4^+) - 8 mg kg-1, P_2O_5 - 30 mg kg^{-1} soil K_2O -120 mg kg^{-1} soil. The following content of the studied HMs was measured (in μg g^{-1} DW): Cd – 0.25, Cu - 22.83, Pb - 16.00, Zn - 46.03. The polluted field near the waste depository of a ferrous metallurgical combine with leached cinnamonic forest soil had the following agrochemical characteristics: pH(H_2O) = 7.94, 10 mg kg^{-1} soil total mobile nitrogen (N-NO_3^- + N-NH_4^+), 36.8 mg kg^{-1} soil P_2O_5, 308 mg kg^{-1}soil K_2O. The content of the studied HMs (μg g^{-1}DW) was as follows: Cd – 14, Cu – 27, Pb – 142 and Zn – 207. As the Bulgarian permissible limit concentrations (PLC) at pH(H_2O) = 7.94 are Cd - 3.0, Cu < 260, Pb < 80 and Zn < 340 μg g^{-1} DW, the soils can be considered as heavily polluted with Cd and Pb only. The content of Cd and Pb exceeded PLC 4.6 and 1.8 times, respectively.

All treatments were arranged in randomized complete block design with three replications. The experimental plot area was 9 m^2 with density 5 plants m^{-2}. Two formulations of foliar fertilizers (Agroleaf®, Scotts Company, Wooster, Ohio, USA) were applied: (1) Agroleaf® total – N:P:K=20:20:20 + microelements (N – NH_4^+ + NO_3^-, P – P_2O_5, K – K_2O), was applied twice during the vegetative growth stage in a 20-day interval until the bud formation phase; (2) Agroleaf® with high P - N:P:K=12:52:5 + microelements, was applied before the blooming stage. Microelements in a chelated form are presented in concentrations: 0.1% Fe, 0.06% Mn, 0.06% Cu, 0.06% Zn, 0.02% B. Agroleaf® was applied by spraying at rates of 0.5 g of product m^{-2} (0.5% solution recommended by Scotts Company).

MDA was estimated according to Heath and Packer (1968). For the H_2O_2 assay the method of Jessup et al. (1994) was used. Low molecular antioxidants were determined in plant extracts as described by Doulis et al. (1997).

The concentrations of reduced (GSH) (Grifith, 1980) and oxidized (GSSG) (Fadzilla et al., 1997) glutathione were determined using the enzyme recycling assay. ASC and dehydroascorbic acid (DHASC) were determined by the method of Foyer et al. (1983). Spectrophotometric quantification of lipid soluble antioxidant capacity, expessed as vitamin E was performed (Prieto et al. 1999). The contents of phenolic compounds (Pfeffer et al., 1998) and flavonoids (Zhishen et al., 1999) in plant tissues were measured spectrophotometrically.

Activities of guaiacol peroxidase (GPO) (EC 1.11.1.7), CAT (EC 1.11.1.6), glutathione peroxidase (GPX) (EC 1.11.1.9), glutathione-*S*-transferase (GST) (EC 2.5.1.18), GR (EC 1.6.4.2), APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4.), DHAR (EC 1.8.5.1.) were assayed by tracing the changes in absorbance (Stancheva et al., 2010). Protein content was determined by the method of Lowry et al. (1951). Total antioxidant capacity (free radicals scavenging activity) was measured from the bleaching of the

purple-colored methanol solution of DPPH• (diphenylpycril-hydrazyl - free stable radical) due to its reduction (Tepe et al., 2006). For heavy metal analysis the plant samples were digested in a solution containing $HNO_3/HClO_4$ (3:1, v/v). The samples were dried by heating at 200°C. The residue was taken up in 25 ml of 1N HCl. Metal concentrations were determined using the inductively-coupled Plasma Mass Spectrometer (CCD Simultaneus ICP OES, Varian, Austria). Saponins were determined by Ivanova et al. (2010).

Data are expressed as means ±SE, where n = 3. Comparison of means was performed by the Fisher LSD test ($P \leq 0.05$) using ANOVA analysis.

RESULTS AND DISCUSSION

Elevated levels of the main contaminants in the above-ground parts of *Tribulus terrestris* plants were observed – 8.3 μg g^{-1} Cd versus 0.6 μg g^{-1} in the controls, 95.4 μg g^{-1} Pb versus 2.4 μg g^{-1} in the controls, which showed their good ability for Cd and Pb accumulation (Fig. 1). In puncture vine grown on polluted and non-polluted soil, Cd and Pb content in roots was higher than in shoots. The lowest Cd content was observed in leaves and stems, whereas the lowest levels of Pb and Zn were in fruits. Zn accumulated in shoots to a higher extent than in roots because of its high mobility. Although Zn did not exceed PLC in the soil, increased

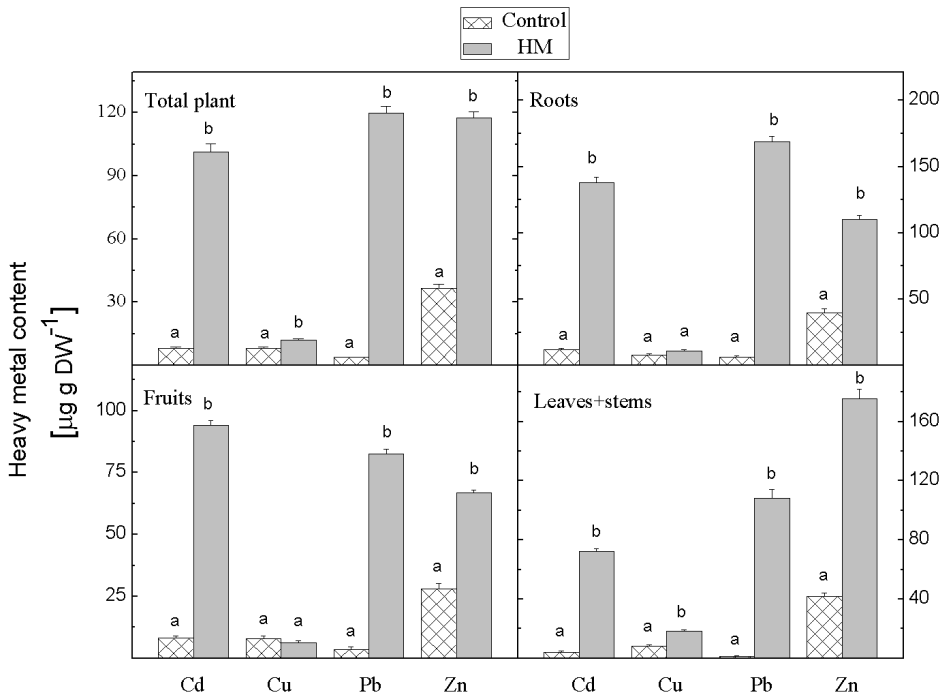

Figure 1. Heavy metal accumulation in organs of *Tribulus terrestris* L. grown on non-polluted control soil (C) and industrially polluted soil (HM). Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test (P ≤ 0.05) after performing ANOVA multifactor analysis.* Cd data were multiplied by 10.

levels of this metal in polluted plants were observed. Copper content in roots and fruits of treated plants did not change significantly compared to control. An increase of Cu was observed in leaves and stems of puncture vine grown on polluted soil. Consequently, *T. terrestris* plants can be classified as accumulators of Cd, Pb and Zn.

In *T. terrestris* shoots and fruits the level of MDA decreased when plants were grown on industrially contaminated soil (Fig. 2). Predominant levels of H_2O_2 content in shoots were observed in comparison with fruits, both in control and polluted plants. Hydrogen peroxide in shoots of stressed plants decreased approximately 2 times in comparison with control, whereas the values in fruits were similar. The increased level of MDA is indicative of elevated lipid peroxides as a result of enhanced ROS production. The lowered level of lipid peroxidation and H_2O_2 concentration in fruits and shoots of *T. terrestris* exposed to HMs indicated that oxidative stress was not strongly expressed. Similar results were obtained for shoots of *T. terrestris* by Stancheva et al. (2011) under glass house conditions. Shoots of control and stressed plants contained much more DHASC than fruits (Fig. 2). The contents of ASC and DHASC decreased significantly both in shoots and fruits of stressed plants. GSH and GSSG concentrations in fruits were higher than in shoots of control plants (Fig. 2). In plants grown on contaminated soil the concentrations

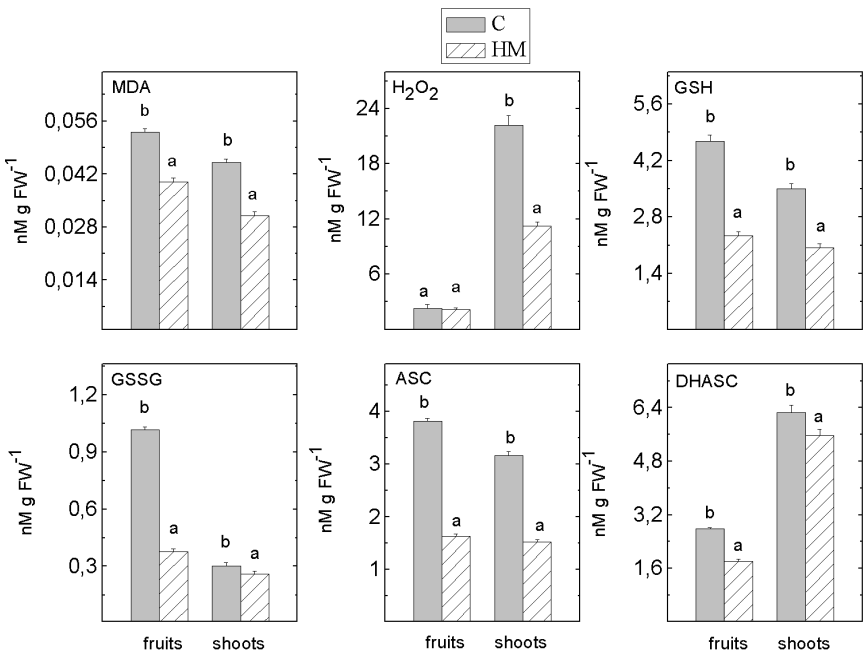

Figure 2. Content of MDA, H_2O_2, GSH and GSSH, ASC and DHASC in fruits and shoots of *Tribulus terrestris* grown on non-polluted control soil (C) and heavy metal polluted soil (HM). Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test **(**$P \leq 0.05$) after performing ANOVA multifactor analysis.

of CSH and GSSG decreased and this trend was more strongly expressed in fruits than in shoots. In contrast, lipid soluble antioxidant capacity expressed as α-tocopherol decreased to a higher extent in shoots than in fruits of plants grown on industrially polluted soil (Fig. 3). Control and contaminated plants were characterized with lower total phenolic and flavonoid contents in fruits than in shoots. The concentrations of total phenols and flavonoids increased in fruits but declined in shoots of plants grown on contaminated soil. The total antioxidant activity (DPPH·) was high both in fruits and shoots of heavy metal stressed plants.

The activities of antioxidant enzymes such as CAT and peroxidases (GPO, GPX and APX) increased in both fruits and shoots of *T. terrestris* due to HM pollution (Fig. 4). CAT and peroxidases competed in removing H_2O_2. Among the enzymes of AGC, the activities of GST, GR, and DHAR decreased in both fruits and shoots of the stressed plants while MDHAR activity decreased only in fruits. According to Procházková and Wilhelmová (2010) the antioxidant systems can be divided into two categories: one that reacts with ROS and keeps them at low levels (peroxidases, SOD, and CAT), and another that regenerates the oxidized antioxidants (MDHAR, DHAR, APX and GR). In puncture vine plants the first antioxidant system was more effective on the account of elevated activity of CAT, GPO and GPX, while only APX regenerated oxidized antioxidant forms.

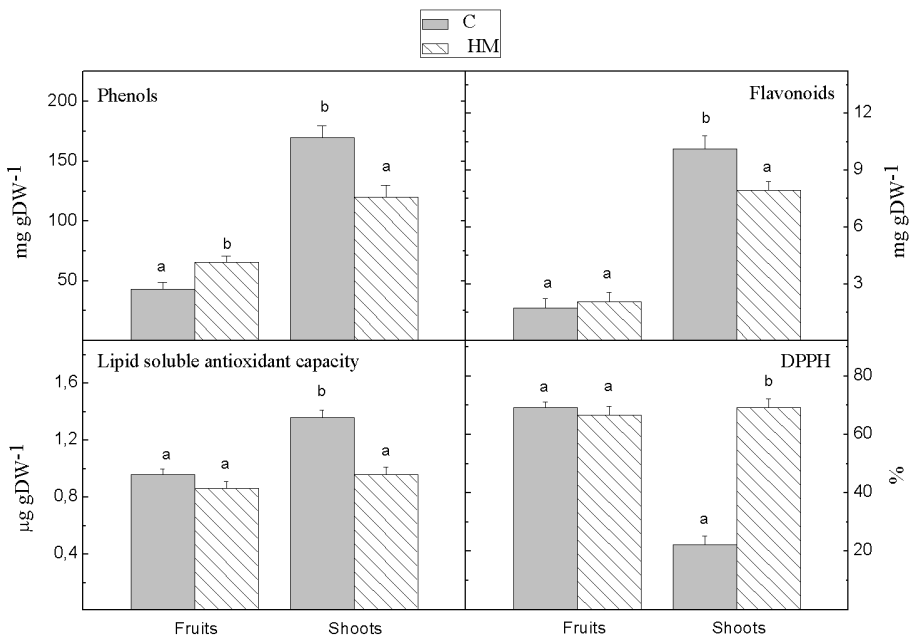

Figure 3. Content of phenols, flavonoids, lipid soluble antioxidants expressed as α-tocopherol and total antioxidant activity (DPPH) in fruits and shoots of *Tribulus terrestris* grown on non-polluted control soil (C) and heavy metal polluted soil (HM). Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test (**P** ≤ 0.05) after performing ANOVA multifactor analysis.

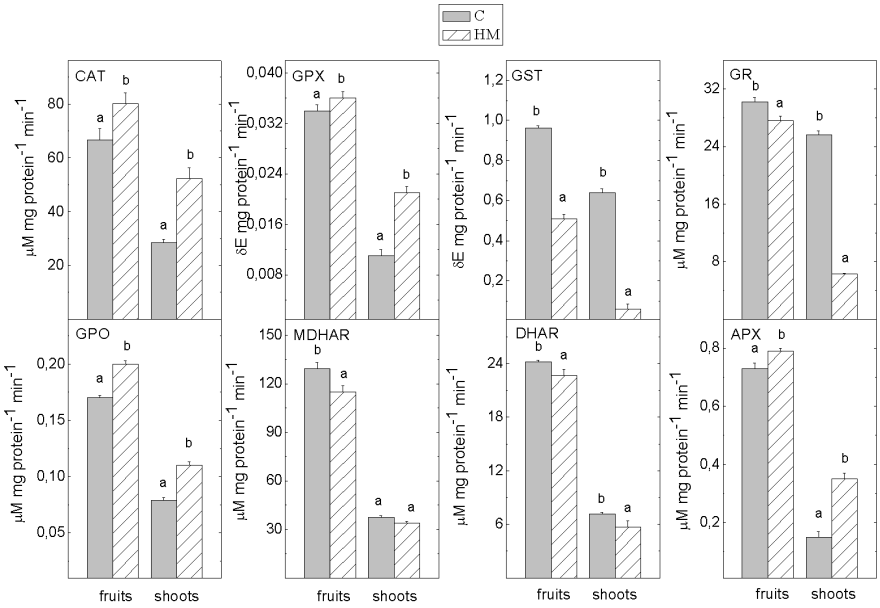

Figure 4. Activity of CAT, GPX, GST and GR, GPO, MDHAR, DHAR and APX in fruits and shoots of *Tribulus terrestris* grown on non-polluted control soil (C) and heavy metal polluted soil (HM). Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

GSH is the major low-molecular nonprotein thiol in plants. Besides serving as a key antioxidant within the cells, GSH acts as a redox buffer, involved in the detoxification of xenobiotics. It also serves as a precursor for the synthesis of phytochelatins, which are crucial in controlling cellular HM concentrations (Foyer et al., 1983). Like ASC and GSH, tocopherols are important antioxidants in the plant cell acting as membrane stabilizers. It is widely believed that the protection of pigments and proteins of photosynthetic systems from oxidative damage caused by ROS is the main function of tocopherols (Sharma et al., 2010). Under conditions of HM exposure, phenolic compounds can act as metal chelators or can directly scavenge ROS. Despite the importance of these low molecular metabolites for antioxidant defense, in *T. terrestris* plants enzymatic components were included in the triggering of a series of defense mechanisms. Hydrogen peroxide can be produced by both non-enzymatic and enzymatic processes in cells. Excessive contents of H_2O_2 could be minimized through the activities of CAT and different peroxidases. Under control conditions, GSSG is reduced efficiently back to GSH by the action of the enzyme GR present in the cytosol, mitochondria and chloroplasts. The decreased levels of GSH could be due to the lowered GR activity in fruits and shoots of heavy metal stressed *T. terrestris* plants. The low levels of MDHAR and DHAR coincided with reduced ASC content in shoots and fruits of plants grown on industrially polluted soil. Under the conditions of our experiment antioxidant defense in response to elevated levels of Cd and Pb

Table 1. Content of biologically active compounds [mg g^{-1}] in *Tribulus terrestris* grown on non-polluted (control) and industrially polluted soils.

Variant	Rutin	Protodioscin	Prototribestin
Control plants	1.96±0.08[b]	9.57±0.42[a]	4.56±0.22[a]
Stressed plants	1.25±0.06[a]	12.19±0.58[b]	6.13±0.29[b]
LSD ($P \leq 0.05$)	0.160	1.15	0.583

Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

in the soil was due mainly to the increased activities of peroxidases such as GPO, APX, GPX and CAT.

The results from determination of biologically active compounds of *T. terrestris* showed that total concentration of furostanol saponins (protodioscin and prototribestin) was higher in the samples from plants grown on industrially polluted soil (Table 1). The content of the flavonol glycoside rutin decreased in these samples. The steroidal saponins are considered to be the factor responsible for biological activity of products derived from *T. terrestris*. Such activity depends on the concentration and the composition of active saponins in the product, which in turn is influenced by the environmental condition of plant growth. Plants grown on soil contaminated with Cd and Pb (far exceeding PLC) were found to contain increased levels of protodioscin and prototribestin.

In summary, the accumulation in high levels of Cd, Pb and Zn in plant tissues caused some changes of antioxidant levels. MDA, GSH and ASC decreased significantly, but the total phenolic concentrations rose in fruits of stressed plants. Antioxidant defense of *T. terrestris* was based mainly on the elevated activities of catalase and peroxidases such as GPO, GPX and APX, while the operation of AGC was not completely effective. Although rutin concentration decreased, the total concentration of other biologically active compounds protodioscin and prototribestin increased significantly in the samples of plants grown on industrially polluted soil.

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