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Original Research

Arsenic Uptake and Phytochelatin Synthesis by Plants from Two Arsenic-Contaminated Sites in Poland

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Abstract

The aim of our study was to assess the strategy developed by terrestrial plants growing in an area contaminated by arsenic to avoid or minimize the toxic effects caused by this element. Eight plant species from two arsenic-contaminated areas were selected for the investigation. Arsenic uptake by different plants was discussed. The speciation analysis of arsenic in plant leaves was performed as well and both inorganic As(III) and As(V) were detected. Moreover, the concentration of phytochelatins in the investigated terrestrial plants was determined. It was noted that the highest concentration of arsenic was found in herb Robert (*Gernium robertianum*) 21 $mg \cdot kg^{-1}$ and common nettle (*Urtica dioica*) 5.3 $mg \cdot kg^{-1}$ in the cases of Złoty Stok and Łomianki, respectively. Phytochelatins were present in all investigated plant species: PC_3 was present in the highest concentration in plants from Złoty Stok (compared to other phytochelatins) while none of the phytochelatins dominated in plants from Łomianki. A correlation between concentrations of phytochelatin and arsenic was found in one of the nettle samples from Łomianki.

Keywords: arsenic speciation, phytochelatins, terrestrial plants, HPLC ICP MS

Introduction

Various chemical forms of arsenic show different levels of toxicity. Inorganic arsenic species are considered the most toxic while such organic arsenic forms like arsenobetaine or arsenosugars are recognized as non-toxic. Due to the high toxic properties of inorganic arsenic compounds, arsenic is an element of environmental concern. The major source of arsenic in the environment is connected with anthropogenic activities [1] (e.g., mines, tanneries, etc.). Increasing levels of arsenic in different environments is occurring in many places all over the world, making it a global issue. Arsenic-contaminated sites include areas of mining activity in Spain ("La Parilla" [2] and "El Cabaco" [3]) or gold and silver mine sites in Korea [4], to name a few. There are also several arsenic-contaminated sites in Poland. One of them is an abandoned gold mine site in Złoty Stok, and the second one includes the area of a former tannery in Łomianki, in the neighborhood of Warsaw. These two areas are the objects of interest in studies described in this paper.

Investigations of some plant species growing in Złoty Stok were described in our previous paper [5, 6]. Yet the study was broadened. In the present paper more plants naturally growing in the contaminated areas in Złoty Stok and, additionally, in Łomianki were characterized in terms of arsenic uptake, accumulation, speciation, and phytochelatin synthesis.

Moreover, plants utilized in currently described studies were collected from different heaps in Złoty Stok, in comparison with plants investigated earlier. In Łomianki site only one plant species – common nettle – was studied

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before and only the accumulation of arsenic by that plant was discussed [7].

In our current paper, six plant species collected in an abandoned gold mine site in Złoty Stok and three plant species collected in the former tannery area in Łomianki were investigated. The following plant species were examined: lady fern (*Athyrium filix-femina*), reed grass (*Calamagrostis arundinacea*), herb Robert (*Geranium robertianum*), woodland strawberry (*Fragaria vesca*), raspberry (*Rubus idaeus*), enchanter's nightshade (*Circaea lutetiana*) from Złoty Stok, and common nettle (*Urtica dioica*), common tansy (*Tanacetum vulgare*), and giant goldenrod (*Solidago gigantea*) from Łomianki.

Moreover, the concentration of phytochelatins in the investigated terrestrial plants was determined. Our attention was focused on that topic as one on the suggested detoxification mechanisms occurring in terrestrial plant species is complexation of xenobiotics by phytochelatins [8]. Phytochelatins are cysteine-rich peptides, which are formed by higher plants to cope with toxic amounts of essential and non-essential ions. The general structure of PCs can be described as:

$$(\gamma\text{-Glu-Cys})_n\text{-Gly}\ (n=2\text{-}11)$$

...where Glu – means glutamic acid, Cys – cysteine, Gly – glycine. The chelating properties of these peptides leads to metal(loid) complexes, which are less toxic than the free metal(loid) ions. This complexation is based on the high affinity of certain element ions, such as arsenite, to the sulfhydryl groups of the PCs located in the cysteine residue [9, 10].

As the studied plants grew in an area contaminated not only by arsenic, additionally in all plant species the contents of Zn, Pb, Cu, Cd, and Cr were examined. In fact the plants probably were subjected to stress caused by the presence of various elements. Therefore, the simple correlation between As content in plants and the synthesis of phytochelatins is not always so unambiguous.

Experimental Procedures

Plants Collection

Plants from Złoty Stok were collected at one of the heaps located in the vicinity of the gold mine. Plants from Łomianki were collected from three sampling points – the first sampling point (1) was located in the direct neighborhood of ruins of the tannery building ruins, the second sampling point (2) 50 meters further and the third (3) 200 m further, respectively, in the vicinity of the local road. All three plant species were collected from the first sampling point while from the second and third sampling only common nettles were collected.

Determination of Total Content of Studied Elements

Total concentrations of arsenic and a few other elements (i.e. Cd, Cu, Pb, Zn, Cr) in leaves of all collected plants were determined by inductively coupled plasma mass spectrometry (ICP MS) according to the procedure described before [11]. Approximately 250 mg of dried plant material and 3 mL of concentrated HNO_3 were placed in PTFE vessels and digested in a microwave system. A three-stage program with the maximum temperature of 200ºC and the maximum microwave power of 1000 W was used (5 min: 20-90ºC; 10 min: 90-170ºC; 30 min: 170-200ºC). Digested samples were transferred to 25 mL volumetric flasks and diluted to the volume with milli-Q water. The digestion of all samples was triplicated. The quantitative determination of arsenic was performed using calibration plot.

Arsenic Speciation Analysis

In the case of samples from Złoty Stok, speciation analysis of arsenic was performed while in the case of samples from Łomianki arsenic speciation analysis was conducted only for nettle samples with the highest arsenic content.

The chosen separation and determination parameters were as follows: mobile phase 0.01 mol·L^{-1} Na_2HPO_4 (80%) and 0.01 mol·L^{-1} NaH_2PO_4 (20%), pH~6; mobile phase flow rate 2 mL·min^{-1}. ICP MS measurements were performed under the following conditions: sweep 5, replicates 5, dwell time 100 ms, ICP RF power 1100 W, lens voltage 8 V, plasma gas flow 15 L·min^{-1}, auxiliary gas flow 1.2 L·min^{-1}, nebulizer gas flow 0.9 L·min^{-1}, measured isotope ^{75}As [11].

Determination of Thiol Compounds

Thiol compounds, including phytochelatins, were determined using HPLC with fluorescence detection [12]. Approximately 300 mg of fresh plant material was placed in a mortar. Volumes of 100 µL of 1 mol·L^{-1} of NaOH, 100 µL of 6 mol·L^{-1} of $NaBH_4$, 1.8 mL of 6.3 mmol·L^{-1} of DTPA and a portion of quartz sand were added to the mortar. After grinding the plant material and centrifuging the obtained solution, 250 µL of the extract was pipetted, and 450 µL of 200 mmol·L^{-1} of HEPPS buffer and 10 µL of 20 mmol·L^{-1} of monobromobimane (mBBr) was added. Derivatization of the thiols was carried out for 30 min in dark test tubes at room temperature. A volume of 300 µL of 1 mol·L^{-1} of methanesulphonic acid (MSA) was added to stop the reaction. A volume of 20 µL of the obtained sample was injected onto the column (C18, Zorbax XDB, 4.6 x 250 mm) and separated at 37ºC. The analysis of the thiols (cysteine Cys, glutathione GSH and three phytochelatins: PC_2, PC_3, and PC_4) was performed using HPLC with fluorescence detection. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN). The following gradient elution program was used: 0-10 min with 8-12% ACN and 10-40 min with 12-35% ACN. The measurements were carried out with an excitation wavelength of 380 nm and an emission wavelength of 470 nm. The identification of the arsenic compounds was performed by comparing the retention times of the obtained peaks to the retention times of standard compounds, and the quantification analysis was based on the standard calibration curves.

Table 1. Determination of elements [mg·kg^{-1} d.w.] and thiols [μmol·kg^{-1} s.m.] in plants collected in Zloty Stok; n≥3.

Analyte	Plant/species					
	Lady Fern *Athyrium filix-femina*	Reed Grass *Calamagrostis arundinacea*	Woodland *Strawberry Fragaria vesca*	Herb Robert *Geranium robertianum*	Raspberry *Rubus idaeus*	Enchanter's Nightshade *Circaea lutetiana*
As	2.1±0.1	4.2±0.2	4.2±0.2	21.3±1.8	1.3±0.1	6.1±0.2
Zn	26.4±1.3	34.7±2.0	60.3±2.9	45.4±3.4	90.3±2.6	59.7±3.2
Cu	7.2±0.5	5.2±0.1	10.1±0.6	7.6±0.5	5.5±0.1	13.0±0.7
Pb	3.7±0.2	4.1±0.2	6.6±0.3	6.1±0.1	1.5±0.1	1.6±0.1
Cys	32.4±10.0	<10	<10	111±10	92.0±25	105±18
GSH	581±139	1157±336	1949±565	1872±393	2329 ±303	1474±206
PC_2	<10	<10	48.1±8.8	82.7±11.6	302±39	76.7±13.0
PC_3	619±87	197±30	356±153	480±96	756±83	347±83
PC_4	70.0±8.4	68.6±6.2	50.8±8.3	130±13	47.6±6.2	93.6±8.4

Cys – cysteine, GSH – glutathione, PC_n – phytochelatins

Results and Discussion

Elements in Plants from Złoty Stok

According to our previous studies it was stated that average concentration of arsenic in soils in Zloty Stok was about 2.0 g·kg^{-1}, while the bioavailability of that element, defined by the efficiency of single extractions with CH_3COOH was 6% [5].

In leaves of plants collected in Złoty Stok, determinations of arsenic and other elements were performed and the results are presented in Table 1. The highest arsenic concentration was determined in leaves of herb Robert. Concentrations of zinc in all investigated plants from Złoty Stok was at the level of dozens mg·kg^{-1} DW, lead was present at the level of several mg·kg^{-1} DW. Cadmium was present in all samples in concentrations lower than the limit of detection ($LOD = x_{blank}+3SD$; <0.05 mg·kg^{-1} DW), but its concentration in raspberry leaves amounted to 0.46±0.06 mg·kg^{-1} DW. Despite relatively high concentrations of arsenic and other elements, leaves of collected plants did not show any morphological alterations, which may be proof that investigated plants developed some detoxification mechanisms that allow them to vegetate in contaminated areas. The mine site in Złoty Stok contain high concentrations of arsenic, but such elements like lead are also present in relatively high amounts. Considering results of determination of thiol compounds in leaves of plants from Złoty Stok (Table 1), it is hard to discover that the presence of high arsenic amounts causes phytochelatin synthesis in the investigated plant species. The presence of phytochelatin PC_3 was noted in leaves of all investigated plant species; moreover, PC_3 was in the highest concentration among other PCs. Phytochelatin PC_4 was also determined in all samples, but its concentration was significantly lower in comparison to PC_3. The highest concentration of PC_2 was determined in raspberry leaves, but the concentration was approximately twice lower compared to the concentration of PC_3. Leaves of lady fern and reed grass did not contain PC_2. The concentration of PC_2 was higher than the concentration of PC_4 only in the case of raspberry. Leaves of lady fern contained the lowest concentration of GSH, while leaves of herb Robert incorporated the highest concentration of cysteine. Concentrations of cysteine in leaves of woodland strawberry and reed grass was lower than the limit of detection (<10 μmol·kg^{-1} DW). Comparing the concentrations of thiol compounds and elements determined in the investigated samples, it is difficult to find any general correlation between them. Similar tendencies are noticeable only between concentrations of Zn and GSH. In all investigated samples the increase of GSH concentration is correlated with the increase of Zn concentration.

Elements in Plants from Łomianki

Based on the studies carried out for soils from Łomianki [13], is was stated that average total As amount is 10 times lower than in Złoty Stok, while the mobility in that case was higher – about 20%. Results of determination of arsenic and other elements in leaves of plants collected in Łomianki are presented in Table 2. Considering the chemicals that were used during tanning, areas of deposition of tannery wastes may contain relatively high concentrations of such elements as arsenic or chromium. Therefore, it may be supposed that plants growing in such areas contain relatively high concentrations of these elements. Besides As and the above-mentioned metals, Cr also was determined.

Nettle leaves collected in the third collecting point contained the highest concentrations of As (5.3 mg·kg^{-1} DW) and Cr (6.8 mg·kg^{-1} DW). Concentrations of As in plants collected from other points was significantly lower and did not exceed 1.6 mg·kg^{-1} DW. Cadmium was present in all

Table 2. Determination of elements [mg·kg^{-1} d.w.] and thiols [μmol·kg^{-1} s.m.] in plants collected in Łomianki; n≥3.

Analyte	Plant/species				
	Common Nettle *Urtica dioica* (1)	Common Nettle *Urtica dioica* (2)	Common Nettle *Urtica dioica* (3)	Giant Goldenrod *Solidago gigantea*	Common Tansy *Tanacetum vulgare*
As	0.67±0.02	1.6±0.1	5.3±0.4	1.6±0.1	0.94±0.07
Zn	27.2±3.4	49.4±3.5	6.4±0.5	65.6±2.1	252±7
Cu	4.2±0.4	4.1±0.1	20.1±1.5	22.0±1.8	10.7±0.4
Pb	1.6±0.1	1.0±0.1	2.2±0.2	2.3±0.2	2.0±0.2
Cr	2.8±0.1	2.1±0.1	6.8±0.4	1.0±0.1	10.7±0.4
Cys	198±80	180±20	279±37	54.4±12.7	<10
GSH	2300±690	2574±397	2047±271	878±83	378±84
PC_2	<10	<10	254±79	<10	<10
PC_3	189±114	145±91	199±57	373±55	638±320
PC_4	36.2±7.8	31.2±7.9	43.7±14.2	639±281	<10

Table 3. Concentrations of arsenic species in aqueous extracts of leaves of plants collected in Złoty Stok and Łomianki determined by HPLC-ICP-MS; C±SD [mg·kg^{-1} DW], extraction efficiencies [%], and column recovery [%]; n≥6.

Plant	Identified As species		Sum of species	As concentration in extracts	Extraction efficiency	Column recovery
	As (III)	As (V)				
Raspberry *Rubus idaeus*	0.11 ±0.01	0.16±0.07	0.27	0.29±0.07	22.4	93
Herb Robert *Geranium robertianum*	0.88±0.06	2.18±0.26	3.06	3.81±0.38	17.9	80
Lady Fern *Athyrium filix-femina*	0.33±0.05	0.25±0.02	0.58	0.72±0.04	34.3	80
Reed Grass *Calamagrostis arundinacea*	0.35±0.01	0.54±0.02	0.89	1.04±0.01	24.7	86
Common Nettle 3 *Urtica dioica*	1.53±0.27	1.13±0.16	2.66	2.80±0.06	52.8	95

samples in concentrations lower than LOD, but its concentration in leaves of common tansy amounted to 0.29±0.06 mg·kg^{-1} DW. The highest concentrations of Cu and Zn were determined in giand goldenrod and common tansy, respectively.

The comparison of concentration of thiol compounds and studied elements is presented in Table 2. Comparing the concentrations of thiol compounds and elements determined in the investigated samples, it is difficult to find out which element present in plant tissues is responsible for induction of phytochelatin synthesis, similarly to the plants collected in Złoty Stok. However, more correlations between concentrations of thiol compounds and investigated elements may be observed in plants from Łomianki, in contrast to plants collected in Złoty Stok. Some hypotheses might be formulated on this basis. Leaves of all common nettle samples collected in Łomianki contain significantly higher concentrations of cysteine and GSH in comparison to concentrations of these compounds in other investigated plant species. On the other hand, concentrations of PC_3 and PC_4 in tissues of giant goldenrod and common tansy significantly exceed the concentration of these compounds in common nettle samples. This might be explained by the fact that GSH is a precursor of phytochelatin synthesis; therefore, higher concentration of phytochelatines is connected with the lower concentration of GSH. Concentrations of PC_3 and PC_4 were similar in all nettle samples. Concentrations of cysteine and GSH were similar in all investigated nettle samples as well. However, concentrations of GSH in common nettle from the third sampling point are a bit lower compared to nettles from other sampling points, which is probably connected with the fact that more GSH was consumed in the synthesis of PC_2. PC_2 was identified only in nettles from the third sampling point, and its presence is connected with the higher arsenic concentration compared to other investigated samples.

Therefore, it is likely that the synthesis of PC_2 was induced by the high As concentration in plant tissues. Concentration of Pb in common nettle (3) is similar to the concentration of Pb in other samples. However, PC_2 was not identified in other samples besides leaves of nettle 3. Therefore, the effect of Pb on PC_2 synthesis in nettle 3 can be excluded. Significantly higher concentrations of Zn and Cu were observed in leaves of giant goldenrod and common tansy compared to common nettle samples. The highest concentration of Zn in common tansy is correlated with the highest concentration of PC_3. The highest concentration of Cu is correlated with the highest concentration of PC_4 in giant goldenrod.

To extend the studies and collect more detailed results, speciation analysis of arsenic was performed for samples with sufficient biomass. The results are presented in Table 3. In all cases arsenic was found in two inorganic As(III) and As(V) forms. For common nettle (3), for which the strongest correlation between As content and PC synthesis was discussed, above, it was found that more than 50% of extracted arsenic is present in the form of arsenites, which may be complexed with phytochelatines [14]. The simultaneous presence of As(III) and PCs in leaves of common nettle (3) could be the indirect proof of occurrence of detoxification by induction of phytochelatin synthesis caused by arsenic.

Recently the knowledge of arsenic speciation in biota has increased due to the advances in analytical chemistry, particularly in the area of ICP MS or ES MS/MS. The complexes of As with glutathione and phytochelatins were identified in *Thunbergia alata* [9, 15], *Holcus lanatus,* and *Pteris cretica* [16], or *Helianthus annuus* [17]. Particular attention is paid to the sample preparation procedure and the stability of arsenic-thiol compounds. Obtaining the reliable results remains a challenge. Although the greatest advantage of the mentioned studies is that of heaving plants growing under control conditions – in greenhouses – usually affected only by arsenic compounds. We have focused our investigation on autochthonic plants growing in the contaminated environment – not only by arsenic. That is why the simple correlation between all the obtained results was not always unambiguous.

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