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Response of *Berberis thunbergii* to heavy metals under urban pollution

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Abstract: Increasing pollution of the environment caused by heavy metals is becoming a significant problem in developing cities. Species and cultivars of plants for urban plantings should exhibit tolerance to these pollutants, and what is even more significant, through their absorption they should reduce the level of environmental contamination. The aim of the research was to determine whether *Berberis thunbergii* (DC.), which was grown in the immediate vicinity of roads, developed mechanisms limiting harmful effects of accumulating heavy metals. The mechanism for heavy metal resistance, involving the generation of phytochelatins (PCs), was investigated in relation to As, Cd, Cr, Co, Cu, Hg, Ni, Pb and Zn accumulation. Levels of thiols, i.e. glutathione (GSH) and phytochelatins (PCs), increased in plants grown in polluted areas in the city of Poznań in comparison to a residential site (control) and it was related to the activity of phytochelatin synthase (PC-synthase) and the accumulation of metals. The results indicate that in *Berberis thunbergii* growing in the polluted urban environment a defense mechanism adapting the plant to potentially adverse conditions was initiated.

Additional key words: phytochelatin synthase, phytochelatins, glutathione, heavy metals

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Introduction

Appropriately designed urban greeneries are used to prevent the formation of snowdrifts on traffic routes, to suppress noise and correct driving conditions being used as warning signs. They also eliminate air pollutants, affect gas exchange and they create a microclimate in cities. Most of them, especially in town centers, are located near traffic routes (Mierzejewska 2004, Wałęza 2006).

Transport contributes to the weakening of plant vegetation. Rapid development of motorization is one of the main reasons of the natural environment contamination. An increase in the number of motor vehicles is a threat to urban green areas also through the introduction of heavy metals into the environment (Indeka and Karaczun 2000).

Over the past 10 years the number of cars in the city of Poznań has increased by about 50% (Radzimski et al. 2010). Heavy metal pollution from automobile sources is released during fuel combustion, abrasive wear of tires, leakage of oils and corrosion of batteries and metallic parts (Akbar et al. 2006).

It is not possible to eliminate or reduce any stress factors in the urban environment, which hinder growth and development, changing the morphology and physiology of plants growing in the direct vicinity of streets. Thus, species and varieties of trees and

bushes tolerant to such stresses need to be carefully selected for urban plantings (Skórkowska 2009).

Berberis thunbergii (DC.) is a very popular garden plant and it is used in urban plant architecture due to its small habit and modest requirements (Seneta and Dolatowski 2005). Barberry bushes can be found in home gardens, near office buildings and industrial facilities, shopping centers, in squares and parks, but they are also grown in the immediate vicinity of roads. Producers selling barberry bushes ensure that they are relatively resistant to soil salinity, frost, periodic drought and urban pollution (Borowski and Latocha 2006).

The mechanism limiting harmful effects of pollutants, including heavy metals, in barberry is not known. In general, to minimize the effects of heavy metal exposure and their accumulation, plants have evolved detoxification mechanisms, which are mainly based on chelation and subcellular metal compartmentation in vacuoles (Clemens 2001). Plant cells in response to increased concentrations of free heavy metal ions produce low-molecular weight thiols, such as glutathione (GSH) and small cysteine-rich peptides – phytochelatins (PCs), with high affinity for toxic metals (Hall 2002). Heavy metals contribute significantly to the generation of excess reactive oxygen species (ROS) and free radicals (FR), which leads to oxidative stress. Scavenging properties of GSH, a key antioxidant, and other molecules such as ascorbic acid, α-tocopherol and phenolic compounds, are involved in defense against free radicals (Cobbett and Goldsbrough 2002, Edwards and Dixon 2005, Foyer and Noctor 2005).

GSH, a major transport and storage reservoir form of nonprotein reduced sulfur (Noctor et al. 2002), is a tripeptide synthesized from L-cysteine, L-glutamic acid and in two ATP-dependent reactions catalysed by γ-glutamyl cysteine synthetase and glutathione synthetase (Foyer and Noctor 2001). GSH is a substrate for phytochelatins, polypeptides with the general structure of $(\gamma\text{-Glu-Cys})_n\text{Gly}$ (n = 2–11) (Freeman et al. 2004). Phytochelatins (PCs) are synthesized non-translationally in a transpeptidation reaction catalyzed by phytochelatin synthase (PC-synthase) (Vatamaniuk et al. 2000). Induction of synthesis occurs by exposure to heavy metals such as Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Bi^{3+} and Au^{3+} (Cobbett 2000, Vatamaniuk et al. 2000). PCs form complexes buffering excess toxic metal ions in the cytosol and subsequently transport them into the vacuole (Hall 2002).

The aim of study was, first, to determine whether the accumulation of heavy metals in barberry was dependent on the plant site within the urban agglomeration. Secondly, it was investigated whether the accumulation of heavy metals activated the tolerance mechanism associated with the induction of phytochelatin synthesis, thus the phytochelatin synthase activity and the level of GSH and PCs were described.

Material and methods

Berberis thunbergii collected from selected areas located in the city of Poznań was investigated. There were 6 sites directly exposed to urban pollution, i.e. near roads, tram tracks and a petrol station, and 2 control sites in residential areas, not surrounded by transport routes. Leaves from at least 4 randomly selected shrubs were collected at the beginning of May, July and September. After being delivered to the laboratory, leaves were surface-washed, and weighted samples were frozen in liquid nitrogen and stored at –18°C. Analyses for each parameter were made for 3 analytical replicates, which were averaged. The mean values for each term involve 6 analyses for polluted sites and 2 for the control ones.

Heavy metal determination

Frozen leaves were dried (72 h) in an electric drier at 105±5°C, ground in a laboratory ball mill equipped with a 0.43 mm sieve and four representative samples (3 g each) were collected. Samples were mineralized in a closed Mars 5 X-press microwave mineralization system by CEM using HNO_3 and H_2O_2. Concentrations of cadmium, chromium, cobalt, copper, lead, nickel and zinc were analyzed by atomic absorption spectrometry with flame and electrothermal atomization using a Spectra 200 AA spectrometer by Varian. The content of arsenic was analyzed by hydride generous atomic absorption spectrometry (HGAAS) and mercury by cold vapor atomic absorption spectrometry (CVAAS). The apparatus was equipped with a system of a four-lamp carousel, a GTA–100 atomizer with programmable temperature within the range of 40–2000°C per second, accurate to 1°C, a Czerny-Turner monochromator and a detector coupled directly with a computer. The deuteron background correction was applied to minimize interference. Analytical curves were prepared on the basis of a series of freshly prepared solutions from individual stock standard solutions at a concentration of 1g dm^{-3} each.

Measurements of thiols and enzyme activity

Glutathione (GSH) and phytochelatins (PCs) were isolated and separated according to Stroiński and Zielezińska (1997), using the method presented by Tukendorf and Rauser (1990). Frozen leaves (0.5 g) were homogenized with an Art-Miccra homogenizer in 2 ml of 0.5 mmol dm^{-3} HCl. The GSH and PCs were estimated, using reverse-phase HPLC (Varian *Pro Star*) with post-column derivatization using Ellman's reagent and measured at A_{405} between 22–28 minutes of elution from the column. The amount of PC was calculated to the GSH standard.

PC-synthase activity was determined according to Grill et al. (1989). Frozen material (1.5 g) was ho-

mogenized in 6 ml of 100 mmol dm^{-3} Tris-HCl extraction buffer (pH 8.0) containing 10 mmol dm^{-3} mercaptoethanol, 2.5 (w/v) polyvinylpolypirolidone, 1 mmol dm^{-3} K_2EDTA and 5% (v/v) glycerol. The homogenate was centrifuged twice for 30 min at 30 000 g at 4°C. The supernatant was collected as crude extract and subjected to ammonium sulfate precipitation (40 and 70% saturation). Pellets were dissolved in 1 ml of 50 mmol dm^{-3} Tris-HCl buffer (pH 8.0) containing 25 mmol dm^{-3} KCl, 10 mmol dm^{-3} mercaptoethanol, 0.5 mmol dm^{-3} K_2EDTA and 20 mmol dm^{-3} $MgCl_2$ and dialyzed overnight. Insoluble material was removed by centrifugation and the supernatant was used to determine PC-synthase activity. The incubation mixture contained in the final volume of 1.2 ml: extract (0.7–1.5 mg protein), 50 mmol dm^{-3} Tris-HCI (pH 8.0), 1 mmol dm^{-3} $MgCl_2$ and 45 mmol dm^{-3} KC1, 10 mmol dm^{-3} mercaptoethanol, 0.2 mmol dm^{-3} gluthatione and 2 mmol dm^{-3} $CdCl_2$. After incubation for 60 min at 35°C, 0.1 ml 0.6 mmol dm^{-3} NaOH containing 1 mg $NaBH_4$ was added. The mixture was neutralized by 0.15 ml 5N HC1. As a product of enzyme reaction, the level of phytochelatins was estimated, as described above. Total protein content was measured according to Bradford (1976).

Statystical analysis

Results were subjected to Anova stastical analysis and the Tukey's HSD multiple range tests, using Statistica 9.0 software. Moreover, correlation coefficients for the relationship between heavy metal content and thiols levels (GSH, PCs) and PC-synthase activity were determined.

Results and Discussion

Heavy metal accumulation

Since the content of analyzed metals was highly variable, the results were divided into 3 groups. These ranges were as follows: for cadmium and cobalt from 0 to 1 μg g^{-1} dry weight (DW) (Fig. 1) for arsenic, chromium, copper, nickel, lead and mercury 0.8 to 5.5 μg g^{-1}DW (Fig. 2) and for zinc 12 to 22 μg g^{-1}DW (Fig. 3). The contents changed during the vegetation period, independently of the plant locality; with the lowest values recorded in May and the highest in September, although the differences were insignificant.

Content of heavy metals were depend on growth position (Table 1). In the polluted areas the level of most metals exceeded by 50–100% their contents at the control sites. The highest accumulation was found for lead, the concentration of which reached 4–5 μg g^{-1}DW, while its level in the plants of residental areas was approx. 1 μg g^{-1}DW.

Heavy metals in urban and industrial areas in all regions of worldwide have been well-recognized as plant contaminants (Samecka-Cymerman and Kempers 1999, Mandal 2006, Wang et al. 2003, Ozaki et al. 2004, Honour et al. 2009, Johansson et al. 2009). Our studies confirm the above reports that the accumulation of toxic metals was dependent on the plant's site within the urban agglomeration.

Essential heavy metals are taken up and accumulated by plants. Some plants are able to accumulate toxic metals, which have no known biological function. In barberry leaves an increase in heavy metal content was observed for microelements, desirable from the point of view of plant growth and development (Zn, Cu, Ni, Co), as well as the level of toxic metals such as Cd, Cr, Pb, As and Hg. In particular, the highest accumulation of Pb was found. Pb originates principally from burning fossil fuels and from vehicle emissions (Singh et al. 1995, Altaf 1997, Pirzada et al. 2009), although Pb levels have been decreasing with the introduction of unleaded fuels.

Metal ions accumulated in plant cells can be responsible for structural and functional changes and as a consequence – for growth inhibition. However, many plants show a high degree of tolerance (Alloway and Ayres 1999, Kabata-Pendias and Pendias 1999). A

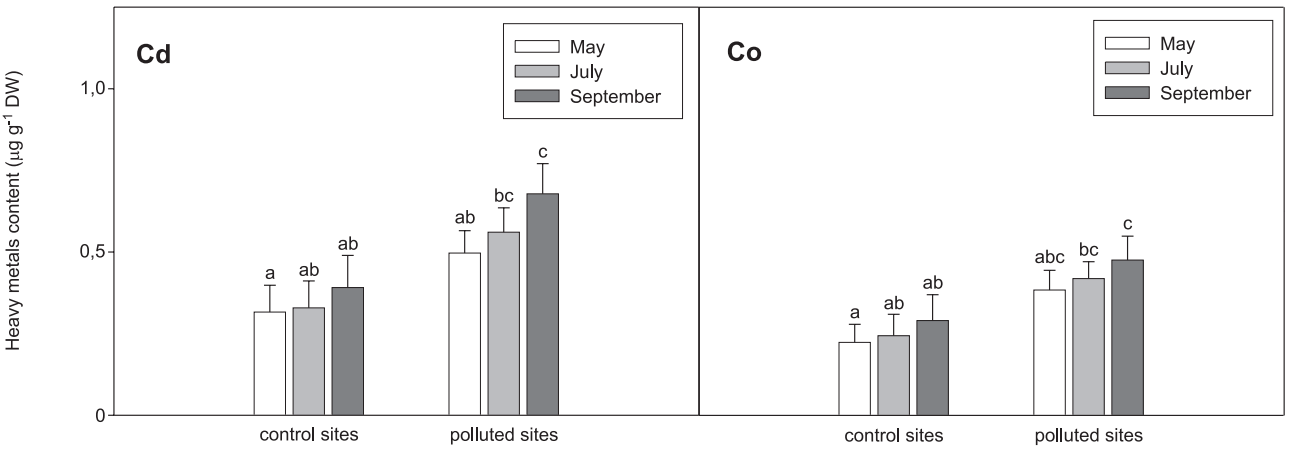

Fig. 1. Cadmium and cobalt accumulation of *B. thunbergii* leaves growing in green area located in the city of Poznań. Values marked with different letters are significantly at $P < 0.05$

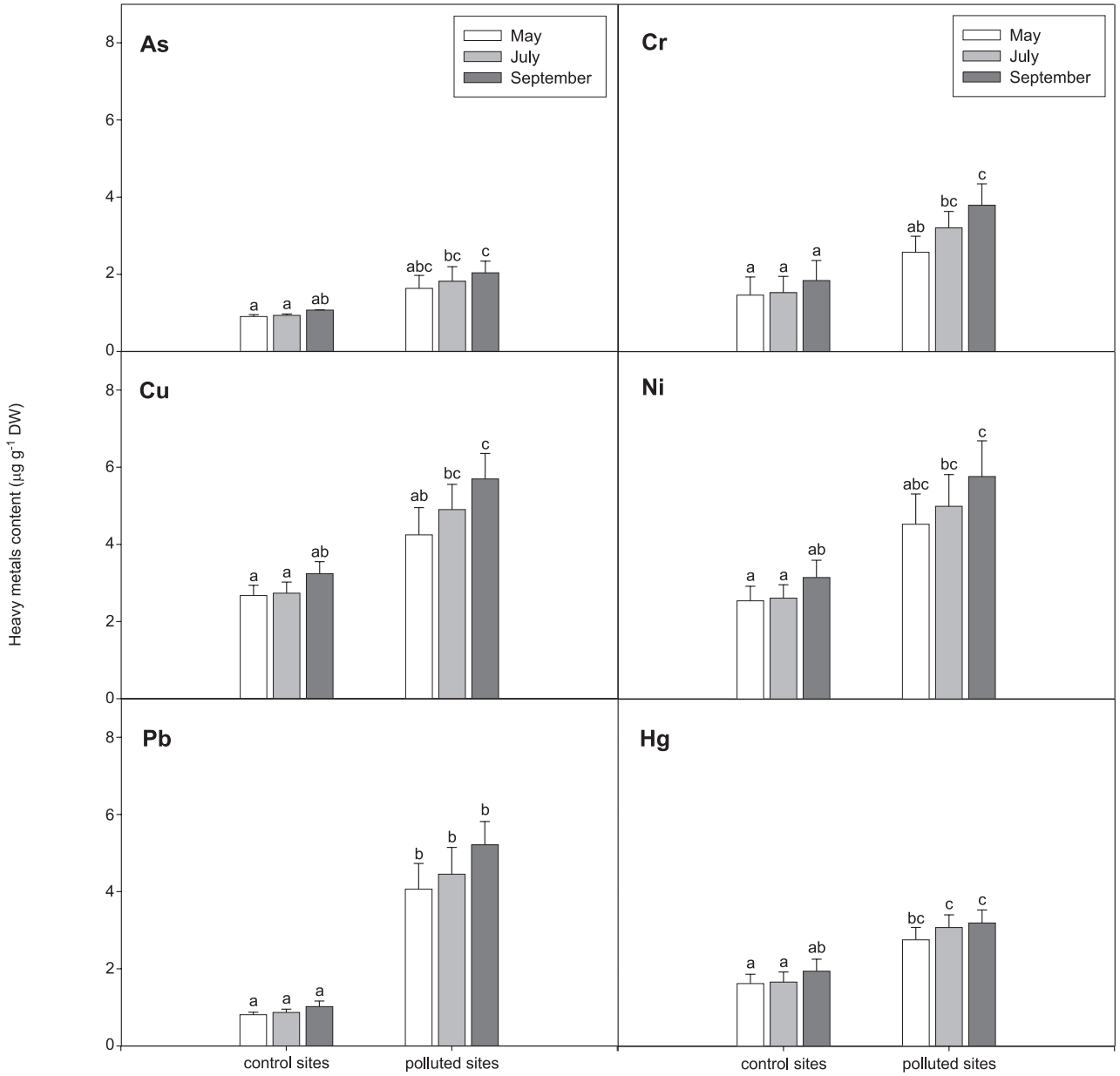

Fig. 2. Arsenic, chromium, cooper, nickel, lead and mercury accumulation in *B. thunbergii* leaves growing in green area located in the city of Poznań. Values marked with different letters are significantly at $P < 0.05$

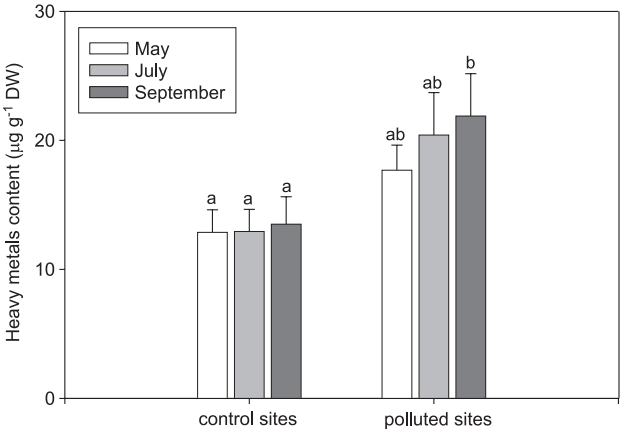

Fig. 3. Zinc accumulation in *B. thunbergii* leaves growing in green area located in the city of Poznań. Values marked with different letters are significantly at $P < 0.05$

similar phenomenon was reported for barberry, since plants did not show visible effects of toxicity, at least under the observed heavy metals concentrations.

Contents of thiols and phytochelatin synthase activity

A common plant tolerance mechanism for heavy metals involves the synthesis of phytochelatins (PCs), low-molecular-weight adverse ions binding proteins, which constituted the subject of our research. Contents of glutathione (GSH), a PCs precursor, and PCs are shown in Figure 4. The GSH level was similar at all sites in the range 50 to 80 nmol g^{-1} fresh weight (FW) of leaves. However its content significantly increased during vegetation period (Table 2).

Table 1. Summary of ANOVA results for heavy metals by site of plant growth and term of material collection

Source of variation	As			Cd			Co		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Site (S)	1	30.46	<0.0001	1	30.31	<0.0001	1	27.67	<0.0001
Term (T)	2	1.13	NS	2	3.22	NS	2	1.98	NS
S × T	2	0.19	NS	2	0.53	NS	2	0.05	NS
Error	18			18			18		
Source of variation	Cr			Cu			Hg		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Site (S)	1	41.48	<0.0001	1	42.41	<0.0001	1	49.54	<0.0001
Term (T)	2	3.55	NS	2	3.47	NS	2	1.37	NS
S × T	2	1.02	NS	2	0.67	NS	2	0.02	NS
Error	18			18			18		
Source of variation	Ni			Pb			Zn		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Site (S)	1	33.88	<0.0001	1	149.54	<0.0001	1	23.89	0.0001
Term (T)	2	1.87	NS	2	1.74	NS	2	0.99	NS
S × T	2	0.21	NS	2	0.84	NS	2	0.57	NS
Error	18			18			18		

NS – non significant.

The concentration of PCs varied over a wide range of values, considering the measurement from May to September and the growth area, i.e. 100 to 2000 nmol g^{-1} FW. The largest differences were found in May and July, when the PCs level was respectively 6- and 2-fold higher in polluted as compared to control sites. In general the PCs content was significantly higher in barberry from polluted areas (Table 2).

Taking into account the above changes, leading to the formation in the cytoplasm of metal-GSH or metal-PCs complexes, which may be transported to the vacuole, heavy metals did not result in metabolic disturbances in barberry. Synthesis of PCs was most frequently accompanied by a simultaneous decrease of GSH (Tukendorf and Rauser 1990, Tukendorf 1993, Cobbett 2000, Ammar et al. 2008). Our previous studies on Cd-induced potato tuber tissues showed that GSH content did not change (Stroiński et al. 2010). Such dependencies were not found in barberry, which may suggest the induction of synthesis for PCs and GSH. High levels of GSH are not only important for PCs synthesis, but can be involved in a reduction of oxidative stress.

PCs are capable of metal binding via sulfhydryl and carboxyl residues (Mudgal 2010). Most studies on the formation of PCs-metal complexes concern Cd ions, but other metals are bound by these oligopeptides as well. Moreover, the presence of heavy metals in the cytosol was a crucial factor for the induction of phytochelatin synthesis. In *Rauvolfia serpentine* cell suspension and in *Rubia tinctorum* root culture the tendency for PCs induction decreased in the following order: Hg>Cd>As>Cu>Ni>Pb>Zn (Grill et al. 1989, Maitani et al. 1996). PCs, in addition to heavy metal detoxification, can be important in the homeostasis of essential metal ion metabolism; how-

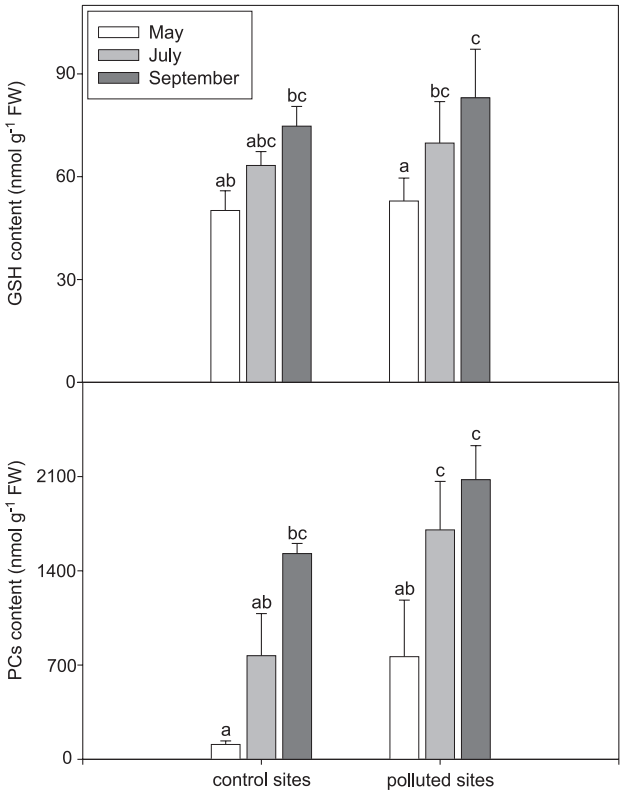

Fig. 4. Thiol content (glutathione and phytochelatins) in *B. thunbergii* leaves growing in green area located in the city of Poznań. Values marked with different letters are significantly at $P < 0.05$

Table 2. Summary of ANOVA results for phytochelatin synthase activity and thiol compounds by site of plant growth and term of material collection

Source of variation	PC-synthase			GSH			PC		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Site (S)	1	32.63	<0.0001	1	2.36	NS	1	22.12	0.0002
Term (T)	2	76.81	<0.0001	2	17.16	<0.0001	2	24.88	<0.0001
S × T	2	0.74	NS	2	0.18	NS	2	0.52	NS
Error	18			18			18		

NS – non significant.

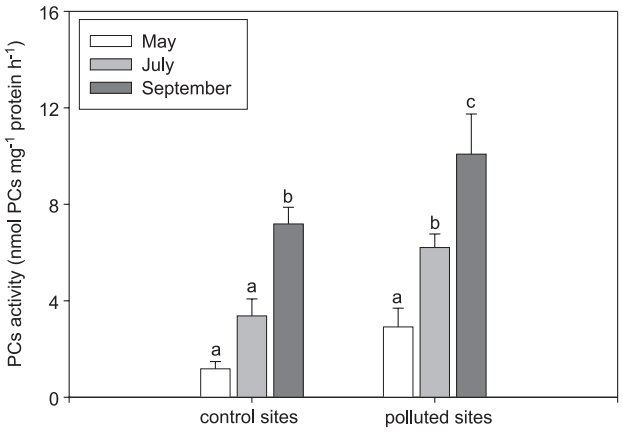

Fig. 5. Phytochelatin synthase activity in *B. thunbergii* leaves growing in green area located in the city of Poznań. Values marked with different letters are significantly at $P < 0.05$

ever, currently no direct evidence is available (Cobbett and Goldsbrough 2002).

Generally, the level of PCs is dependent on the activity of phytochelatin synthase (PC-synthase). Activation of PC-synthase found in barberry leaves was related to the accumulation of those heavy metals binding proteins (Fig. 5). In May at the control location its activity was 1.2 nmol PCs mg^{-1}protein h^{-1} and it increased gradually (3.5 in July and 7.5 in September). In case of polluted sites its activity amounted to: 2.5 nmol PCs mg^{-1} protein h^{-1} in May, 6.4 in July and more than 10 nmol in September. In that case the activity in the contaminated locations was always approx. 2-fold higher than at the control sites. The PC-Synthase activity was depend on plant growth site and term of material collection (Table 2). Moreover, positive correlations were found between heavy metal contents, the phytochelatins synthase activity and the level of thiols (Table 3).

Table 3. Correlation coefficient between heavy metals content, phytochelatin synthase activity and thiol levels

Metal content	PC-synthase activity	GSH level	PCs level
	N = 24		
As	0.4834	0.3711	0.4966
Cd	0.5729	0.3655	0.6125
Co	0.5964	0.4281	0.6827
Cr	0.6154	0.4537	0.6243
Cu	0.6083	0.4768	0.5765
Hg	0.4825	0.2955	0.5919
Ni	0.5746	0.3951	0.6332
Pb	0.4964	0.3286	0.5038
Zn	0.5108	0.3464	0.4526

The PC-synthase activation mechanism is relatively non-specific, although some metals are more effective than others. Activation of the purified enzyme from *Silene cucubalus* cell suspension cultures by Hg was only 27% of the equimolar effect of Cd (Grill et al. 1989). PC-synthase activity has been detected under heavy metal treatment and is induced by metals in many plant species (Howden et al. 1995, Klapheck et al. 1995, Chen et al. 1997, Stroiński and Zielezińska 2001, Stroiński et al. 2010).

In summary, our study cannot determine which element was the most important in phytochelatin generation and which of them formed PCs complexes. However, the results indicate that in *Berberis thunbergii* growing in a polluted urban environment a defense mechanism adapting the plant to potentially adverse conditions was initiated.

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