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Research Article

CONTENT OF REDUCED GLUTATHIONE FORM AS A BIOMARKER OF OXIDATIVE STRESS IN SPINACH PLANTS GROWING IN SOIL CONTAMINATED WITH ZINC

Monika Arasimowicz[1], Barbara Wiśniowska-Kielian[2], Marcin Niemiec[3]

[1] Department of Agricultural and Environmental Chemistry, University of Agriculture, 21 A. Mickiewicza Ave., 31–120 Kraków, Poland, e-mail: komorowska.monika@interia.pl

[2] Department of Agricultural and Environmental Chemistry, University of Agriculture, 21 A. Mickiewicza Ave., 31–120 Kraków, Poland, e-mail: e-mail: rrkielia@cyf-kr.edu.pl

[3] Department of Agricultural and Environmental Chemistry, University of Agriculture, 21 A. Mickiewicza Ave., 31–120 Kraków, Poland, e-mail: niemiecm@tlen.pl

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ABSTRACT
The aim of the paper is the assessment of the efficiency of anti-oxidative system in spinach plants growing in the substratum polluted with zinc. The assessment was conducted on the basis of changes of reduced glutathione (GSH) form concentration in the plant aboveground organs. Spinach plants, 'Matador' c.v., were cultivated in soils contaminated with zinc in two pot experiments conducted in 2010 and 2011. The experimental substratum was light, slightly soil with granulometric composition of sandy silt loam. Zinc in the acetate form, was supplied to the soil in four doses corresponded to this metal critical concentrations in soil with 0, I, II and III degrees of pollution with this element according to IUNG classification. Simultaneously, the control with natural Zn content in soil was maintained. Zn concentrations in spinach ranged from 412.8 to 1722 mg · kg^{-1} d.m. and increased with growing degree of substratum pollution with this element. Over the course of the vegetation period the content of Zn in plants was generally greater. GSH content in spinach grown in both years of experiments fluctuated from 31.70 to 238 µg · g^{-1} f.m. The biggest content of this compound in spinach was stated in the initial phase of plants growth. The plants tolerated only the first two Zn doses supplied to the substratum. Spinach growing in the objects where zinc additions to the soil equalled II and III degree of substratum pollution died shortly after germination. The plants from these objects in the initial growth phase contained significantly less GSH than spinach from the objects with two first degrees (0 and I) of substratum pollution with zinc or from the control. The content of reduced glutathione form in spinach is a good biomarker of oxidative stress caused by zinc presence in plants. Synthesis of a bigger amount of GSH conditions spinach plant resistance to over the norm zinc content in soil. The efficiency of anti-oxidative system in spinach is bigger in the initial phase of this plant growth.

Keywords: environmental stress, glutathione, biomarker of oxidative stress, soil pollution, zinc.

INTRODUCTION

Heavy metals cause oxidative stress in plant organism visible as forming of over the norm amounts of reactive oxygen species (ROS) and free radicals (FR) in cells [2, 13]. The course of this stress in plant cells is affected by the activity of their anti-oxidative system, composed of anti-oxidative enzymes and low molecular, usually nonspecific, antioxidants [8].

One of the nonspecific antioxidants is glutathione, whose molecule is a polymer of three amino acids: glutamic acid, cysteine and glycine [6, 14, 16]. In the presence of ballast trace elements and with an excess of elements crucial for the cell, glutathione fulfils a double function. The compounds decreases oxidative stress: directly by scavenging free radicals and improving the efficiency of the anti-oxidative system through regeneration of other anti-oxidative compounds, but also indirectly by binding these element ions, which leads to their disabling [16]. Moreover, glutathione is a precursor for phytochelatins – specific non-enzymatic protein compounds able to bind heavy metal ions [9, 12].

The paper aimed to assess the disturbances of the metabolism of spinach plants growing in zinc polluted substratum and estimation of anti-oxidative system efficiency in development of spinach plants growing in Zn polluted soil. The assessment was conducted on the basis of changes in GSH content in the aboveground organs of this plant in three subsequent phases of its development: intensive growth, flowering and seed setting.

MATERIAL AND METHODS

In 2010 and 2011 two pot experiments were conducted in a vegetation hall of the University of Agriculture in Krakow, situated at the experimental station in Mydlniki. The substratum for the experiment was taken from experimental plots in the area of this station. Before the experiment outset analyses of the soil substratum were conducted and the following physicochemical properties were determined with methods commonly used in agro-chemical laboratories:

- granulometric composition – Bouyoucose-Casagrande method in Proszynski's modification,
- maximum capillary water capacity,
- pH in suspension in water and in 1 mol · dm^{-3} KCl solution,
- hydrolytic acidity – Kappen's method,
- total nitrogen content – Kjeldahl's distilling method,
- organic carbon content – Tiurin's method,
- contents of available forms of potassium and phosphorus – Egner-Riehm's method,
- total macroelements and trace metals content after sample decomposition in a mixture of nitric and chloric(VII) acids (3:2; *v*/*v*) following organic matter mineralization in a muffle furnace at 500 ºC [10].

Results are presented in Tables 1, 2 and 3.

The experimental soil was light, slightly acid, contained average amount of organic matter and total nitrogen, and had high content of available phosphorus and very high content of available potassium. The content of Fe and Pb corresponded to their average contents in surface levels of light soils, amounting: 6400 mg Fe and 13 mg Pb · kg^{-1}. Soil content of Mn, Zn, Ni, Cr and Cd were little higher than averages, amounting to: 282 mg Mn, 31 mg Zn, 5.9 mg Ni, 11 mg Cr and 0.21 mg Cd · kg^{-1}, and above 2 times higher than average Cu content equals 6.2 mg Cu · kg^{-1} [7]. Trace el-

Table 1. Texture of experimental soil

Fraction diameter [mm]	1.0 – 0.1	0.1 – 0.05	0.05 – 0.02	0.02 – 0.006	0.006 – 0.002	< 0.002
Share of fraction [%]	45	11	23	13	5	3
Agronomic category	sandy silt loam					

Table 2. Basic physicochemical properties of soil used in the experiments

Water capacity [%]	pH		Hh [mmol$^{(+)}$ · kg^{-1}]	Organic C	Total N	P_2O_5	K_2O
	KCl*	H_2O		[g · kg^{-1}]		acc. to Egner-Riehm [mg · kg^{-1}]	
33.34	5.75	6.25	9.70	7.50	1.22	157.1	281.6

Explanation: 1 mol · dm^{-3} KCl solution

Tabela 3. Total content of macroelements and trace elements in experimental soil

Macroelements [g · kg^{-1}]	Ca		Mg		K	Na	P	
	1.384		9.913		0.888	0.361	0.443	
Trace elements [mg · kg^{-1}]	Fe	Mn	Zn	Cu	Ni	Cr	Pb	Cd
	4921	283.3	39.23	14.35	6.637	11.21	0.930	0.266
Degree of pollution [7]	-	-	0º	0º	0º	0º	0º	0º

ement contents were lower than limit values for natural heavy metals content in surface levels of light soils calculated on the basis their geometric means and geometric standard deviations amounting: 13200 mg Fe, 506 mg Mn, 65 mg Zn, 13.0 mg Ni, 27 mg Cr, 24.5 mg Pb and 0.44 mg Cd $\cdot$ kg^{-1}, and higher than limit for Cd, equals 12.4 mg Cu [7]. Taking into consideration degrees of soil pollution elaborated by the Institute of Soil Science and Plant Cultivation (IUNG) [7] heavy metal contents in soil were natural.

The soil preparation for the experiment comprised its drying in the air, crushing and sifting. Subsequently each pot was filled with 5 kg of soil. A week before plant sowing uniform basic fertilization: 1 g N in the form of ammonium nitrate, 0.25 g P as sodium dihydrogen(V) and 1 g K as potassium chloride, was applied to each pot. Simultaneously, zinc in the form of acetate was added to the soil according to the experiments scheme (Table 4).

Table 4. Scheme of pot experiments

Zinc addition to the substratum	
Degree of contamination acc. to criteria of IUNG [7]	[mg Zn $\cdot$ kg^{-1}]
Control	Natural content
0°	50
I°	100
II°	300
III°	700

Zn was added to the soil in amounts corresponded to critical values of the subsequent degrees of soil pollution with this element as suggested by the Institute of Soil Science and Plant Cultivation [7]. Basic fertilization and zinc supplements were applied as solutions of pure for analysis salts. Spinach, 'Matador' c.v., was cultivated as a test plant.

Reduced form of glutathione (GSH) was determined in compliance with the method described by Gurie [4] with modifications. Glutathione was extracted from plant tissues with a mixture of ethylenediaminetetraacetic acid and trichloroacetic acid (EDTA-TCA). In order to bring pH solution to the value of c.a. 7.0, K-phosphate buffer with pH = 7.0 was used. The content of reduced glutathione was assessed by spectrophotometer using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) – Ellman reagent in Beckman UV/VIS PU 6400 apparatus. The solution extinction was measured at the wavelength λ = 412 nm. Glutathione concentration in the plant biomass was calculated on the basis of the values read from the standard curve. Absorbance of the plant homogenate, which absorbed a part of the radiation, was measured as a blind sample.

Zinc content in spinach aboveground parts was assessed using ICP-AES method after wet mineralization in a mixture of HNO_3 and H_2O_2 acids (6:1, *v*/*v*) in a closed system in a Multiwave 3000 microwave oven (Anton Paar). The solution method was selected for the plants and conducted basing on application instructions of the microwave system manufacturer.

Contents of glutathione, ascorbic acid and nickel were assessed in spinach aboveground parts in three phases of development: intensive growth, flowering and seed setting.

The significance of differences in the analysed chemical compound concentrations in the test plants growing in substrata with various degrees of zinc pollution and in successive vegetation phases were assessed using t-Student test at the significance level $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Zinc concentrations in spinach growing on the control objects of experiments in the 2010 and 2011 were from 412.8 to 552.5 mg $\cdot$ kg^{-1} d.m. (Table 5).

The first Zn supplement supplied to the substratum, corresponding to 0 degree of soil pollution (50 mg $\cdot$ kg^{-1}) caused over two-fold, while the second equal to I degree (100 mg $\cdot$ kg^{-1}) brought about a three-fold increase in the content of this element in spinach aboveground parts in comparison with the control. Bigger additions of zinc to the substratum (the II and III degree of pollution) caused an inhibition of germination and growth of spinach plants (Figure 1).

Zn content in plants from individual objects did not change significantly during their growth (Table 5). According to Yanqun et al [18] zinc accumulation in plants depends on their species.

The highest concentrations of glutathione, irrespective of the applied metal, were registered in the first phase of plant growth. With progressing vegetation glutathione content in the aboveground parts was decreasing and in the last investigated phase was twice or three times lower than in the first (Table 6).

Table 5. Zinc contents in biomass of spinach in individual phases of plants development [mg Zn · kg-1 d.m.]

Phase of plant development	Zinc addition acc. to degree of substratum contamination				
	control	0° *	I°	II°	III°
2010					
I phase	505.8a **	1013.4a	1341.4a	lack of plant material for analyses	
II phase	531.2a	1152.4b	1346.4a		
III phase	552.5a	1081.1ab	1625.4a		
2011					
I phase	412.8a	1006.7ab	1301.6a	lack of plant material for analyses	
II phase	560.8b	1127.4b	1704.3b		
III phase	511.6b	968.9a	1722.1b		

Explanation: * – concerns Tables 5–7 and Figures 1–3: 0°, I°, II°, III° – Zinc additions corresponded to degree of successive substratum contamination acc. to criteria of IUNG; ** – concerns Tables 5–7: different letters indicate significant differences depending on zinc additions in relation to control object and objects with lower degree of pollution, at α ≤ 0.05.

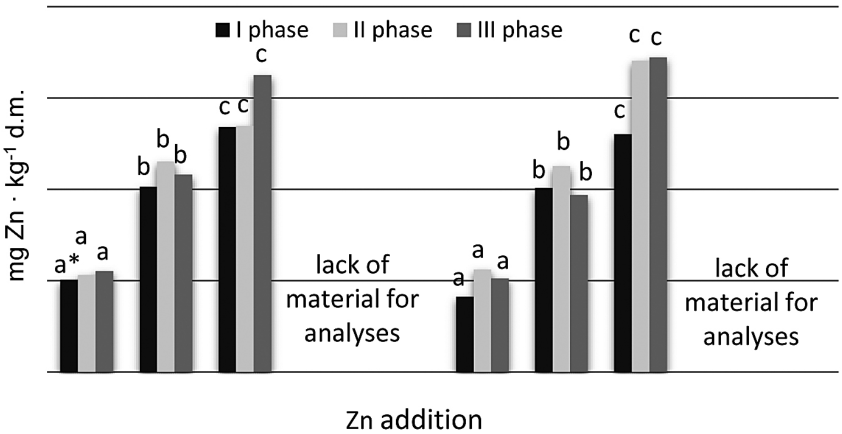

Figure 1. Zinc contents [mg Zn · kg-1 d.m.] in aboveground biomass of spinach in individual phases of plants development depending on this metal addition

Explanation: * concerns Figures 1–3: different letters indicate significant differences depending on nickel additions in relation to control object and objects with lower degree of pollution, at α ≤ 0.05

Table 6. Glutathione content in biomass of spinach in individual phases of plants development [µg GSH · g-1 f.m.]

Phase of plant development	Zinc addition acc. to degree of substratum contamination				
	control	0°	I°	II°	III°
2010					
I phase	78.92a	176.83a	180.14a	94.37	59.56
II phase	64.47b	96.83b	190.24a	–	–
III phase	31.70c	59.63c	142.62b	–	–
2011					
I phase	87.33a	132.32a	238.03a	129.67	99.58
II phase	90.76a	130.46a	201.76a	–	–
III phase	59.01b	128.90a	226.81a	–	–

Glutathione concentrations in the aboveground parts of spinach growing in the objects with zinc supplement in the substratum ranged in both years of the experiment from 59.56 to 238.03 μg · g^{-1} f.m. (Figure 2). Guri [4] assessed approximate glutathione content in fresh mass of pea (*Phaseolum vulgaris* L.) a variety sensitive to ozone effect and exposed to this stressor.

In the Authors' own research, in both years of the experiments, glutathione content in spinach increased significantly in response to Zn additions corresponded to 0 and I degree of substratum pollution in relation to the control. After application of Zn in the amounts corresponding to II and III degree of pollution, glutathione concentration in spinach in 2011 decreased markedly in comparison with the previous objects, whereas in 2010 its content declined significantly also in relation to the control. Agarwal [1] in the research on the effect of various UV radiation intensity on antioxidant content in plant tissue determined a notable decrease in glutathione (GSH) content at two-fold increase in the stressor intensity. Two larger zinc additions (300 and 700 mg · kg^{-1} soil d.m.) initially caused inhibition of spinach growth and then the plant die back. Spinach plants which contained lesser quantities of glutathione than the plants from the control and less polluted objects, did not tolerate high zinc concentrations. As a result of high toxicity of the highest applied supplements of this metal, the plants after germination first grew poorly and then died. Presence of reduced form of glutathione and possibility of its quota regeneration in a plant cell is a condition of tolerating high concentrations of trace elements [5]. Heavy metals present in a plant cell cause numerous disturbances and impairments of all live processes. Each organism has its own specific limit of tolerance to individual stressors. When stressor intensity is too high and disturbances due to its presence are too serious, the tolerance mechanisms fail and the cell dies [15]. Low content of reduced glutathione in the plants growing in the substratum with Zn supplements corresponding to II and III degree of pollution was connected with too high concentration of zinc ions in cells. Free metal ions disable functions of enzymes, among others glutathione reductase which is responsible for reducing oxidized glutathione and therefore for recovering its abilities for these ions chelating [11, 17]. Moreover, free ions of toxic elements lead to formation of reactive oxygen species which injure and damage lipids, proteins, carbohydrates and nucleic acids, disturbing the proper metabolism of a living cell [3]. Spinach plants growing in the other objects with Zn supplements, in the 2nd and 3rd development phase, despite smaller amount of produced biomass in comparison with the control plants, tolerated the applied metal supplements. In the 2nd and 3rd phase of growth glutathione content in the plants tolerating Zn quantities added to the soil and growing in the objects with increasing substratum pollution with this metal, was significantly higher than in the control plants.

In the second studied development phase (flowering period) glutathione content in spinach was comparable or smaller than in the analogous objects in the first phase. In the second and third plant development phase glutathione concentrations were markedly higher in the plants growing in substrata with increasing degree of zinc pollution. In the third phase of plant growth, this compound concentration in spinach was generally lower than in the first phase (Table 6). The tendency was more pronounced in 2010.

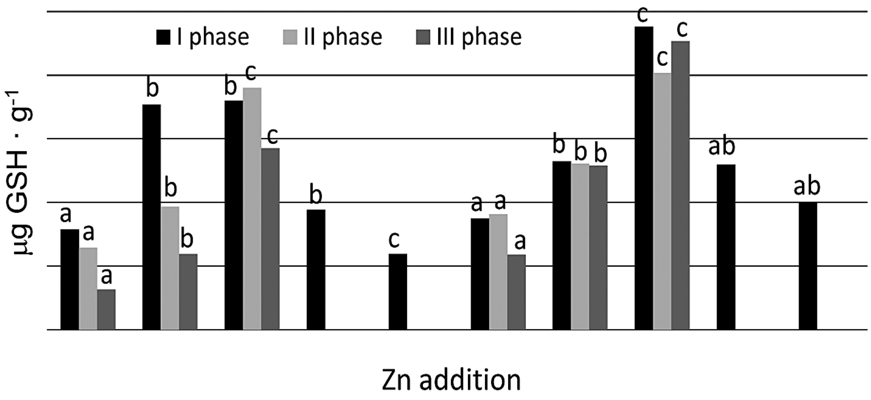

Figure 2. Glutathione content [μg GSH · g^{-1} f.m.] in aboveground parts of spinach in individual phases of development depending on zinc addition

CONCLUSION

The following conclusions may be drawn on the basis of the obtained research results:

1. Spinach plants absorb Zn proportionately to its content in the substratum.
2. Excessive amounts of Zn are tolerated by spinach plants, 'Matador' c.v., greatly owing to the mechanisms connected with glutathione synthesis. There is the tolerance threshold for this metal in plants, above which GSH is no longer synthesised in the amounts sufficient for tolerating the effects of this metal ions presence in cells.
3. The anti-oxidative system in the plant cells functions more efficiently at the initial stage of spinach vegetation, as has been evidenced by much higher glutathione contents in the first phase of growth. At later stages of plant development successively decreasing amounts of this antioxidant are observed.
4. The content of reduced form of glutathione (GSH) in spinach plant biomass is a good indicator (biomarker) of stress caused by Excess of Zn in the substratum.

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