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Monika ARASIMOWICZ[1*], Barbara WIŚNIOWSKA-KIELIAN[1]
and Marcin NIEMIEC[1]

EFFICIENCY OF ANTIOXIDATIVE SYSTEM IN SPINACH PLANTS GROWING IN SOIL CONTAMINATED WITH NICKEL

SPRAWNOŚĆ SYSTEMU ANTYOKSYDACYJNEGO ROŚLIN SZPINAKU ROSNĄCYCH W GLEBIE ZANIECZYSZCZONEJ NIKLEM

Abstract: The paper attempted to assess the activity of antioxidative system in cells of spinach plant, 'Matador' c.v., growing in the soil contaminated with Ni. Plant material for analyses was obtained from two pot experiments conducted in 2010 and 2011 in the vegetation hall of the Experimental Station of the University of Agriculture in Krakow. Ni content in the plant aboveground parts was assessed by ICP-ES method, contents of reduced glutathione form by colorimetry and ascorbic acid by titrimetric method.

Nickel content in spinach aboveground parts ranged from 2.00 to 204.5 mg · kg^{-1} d.m. and increased with growing substratum pollution with this element and usually with plant age. The plants contained from 31 to 238 μg GSH · g^{-1} f.m. In the first three objects with 0^o, I^o and II^o degree of substratum pollution according to IUNG classification, this antioxidant contents were higher in comparison with its amount in plants from the control and objects with lower degree of pollution. In the object with the highest nickel dose application, GSH content in plants decreased significantly in comparison with plants from the other objects, while the plants on this object died shortly after germination. Ascorbic acid content in spinach in the both years of experiments ranged from 24.13 to 73.09 mg · 100 g^{-1} f.m. and increased in plants from the successive objects with growing substratum contamination with nickel.

In the first phase of growth spinach plants contained generally much more of GSH and AsA, which indicated much better efficiency of the antioxidative system at the initial period of growth.

Keywords: antioxidative system, glutathione, ascorbic acid, nickel, spinach

Introduction

Glutathione (GSH) is a tripeptide composed of three amino acids residues: glutamic acid, cysteine and glycine. In a plant cell the compound is located in the cytosol,

[1] Department of Agricultural and Environmental Chemistry, University of Agriculture in Krakow, al. A. Mickiewicza 21, 31–120 Kraków, Poland, phone: +48 12 662 43 47, fax +48 12 662 43 41, email: komorowska.monika@interia.pl

* Corresponding author: komorowska.monika@interia.pl

chloroplasts, endoplasmatic reticulum, vaculoa and mitochondria [1]. Among the numerous functions of this compound, cell redox regulation and antioxidative buffering are regarded as antioxidative properties [2].

Organic acids, including ascorbic acid (vitamin C) (AsA) also possess antioxidative properties [3]. Ascorbic acid is the most important peroxide scavenger in a plant cell [4, 5]. The compound is present in an apoplast, cytosol, plastids and in vacuola of a plant cell. Ascorbate also occurs in cells in two forms: reduced, as L-ascorbic acid (AsA) and oxidized as monodehydroascorbate radical and monodehydroascorbate (DHA) [6]. Ascorbate is a strong antioxidant reacting with superoxide radical anion, hydrogen peroxide, oxygen singlet and organic peroxide radicals [7]. Antioxidative activity of ascorbate relies also on its participation in the reactions of α-tocopherol and β-carotene regeneration from their radical forms [8, 9] and regulation of the redox potential [10]. Moreover, the compound is the cofactor of many enzymes, particularly those containing the transition metal ions [11]. AsA is an electron donor in the reactions catalysed by ascorbate peroxidase (APX) and stimulates expression of APX coding genes [12]. Ascorbate and glutathione are considered the main antioxidants in plant cells and buffers of oxidoreductive reactions, simultaneously performing key functions in the growth and development, and plant response to stressors in the environment [6, 13]. Ascorbate and glutathione are a part of a complex and complicated antioxidative system in plants [14]. Functioning of these compounds in the antioxidative system complement each other in the Halliwell-Asada ascorbate-glutathione cycle (Fig. 1).

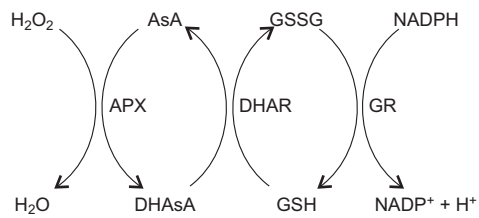

Fig. 1. Scheme of the Halliwell-Asada ascorbate-glutathione cycle [15]

The studies aimed to assess the efficiency of antioxidative system in spinach plants growing in conditions of Ni contaminated substratum on the basis of changes in the content of two basic antioxidants of plant cells: glutathione and ascorbic acid.

Material and methods

Two pot experiments were conducted in 2010 and 2011 in a vegetation hall of the Experimental Station of the University of Agriculture in Krakow, situated in Krakow--Mydlniki. The experimental substratum was obtained from the plots located in the area of the station. Prior to the experiment outset the soil material was analysed and its basic physicochemical properties were determined (Table 1–3).

Table 1

Basic physicochemical properties of soil used in the experiments

pH		Hh	C_{org}	N_{tot}	P_2O_5	K_2O	Water capacity
KCl*	H_2O				acc. to Egner-Riehm		
		$[mmol^{(+)} \cdot kg^{-1}]$	$[g \cdot kg^{-1}]$		$[mg \cdot kg^{-1}]$		[%]
5.75	6.25	9.70	7.50	1.22	157.1	281.6	33.34

* 1 mol · dm^{-3}.

Table 2

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					

Tabela 3

Total content of macroelements and trace elements in experimental soil

Macroelements	Ca	Mg		K		Na		P
$[g \cdot kg^{-1}]$	1.384	9.913		0.888		0.361		0.443
Trace elements	Fe	Mn	Zn	Cu	Ni	Cr	Pb	Cd
$[mg \cdot kg^{-1}]$	4921	283.3	39.23	14.35	6.637	11.21	0.930	0.266
Degree of pollution [16]	—	—	0^o	0^o	0^o	0^o	0^o	0^o

The experimental substratum was light, slightly acid soil, with average content of organic matter and total nitrogen, high content of available phosphorus and very high content of available potassium. Content of Fe and Pb corresponded to average contents in surface levels of light soils, Mn, Zn, Ni, Cr and Cd contents were little higher than averages, and Cu content was above twice higher than average [16]. Fe, Mn, Zn, Ni, Cr, Pb and Cd contents were lower than limit values for natural these metals content in surface levels of light soils calculated on the basis their geometric means and geometric standard deviations, and higher than limit for Cu [16]. According to degrees of soil pollution elaborated by the Institute of Soil Science and Plant Cultivation (IUNG) [16] heavy metal contents in soil were natural.

The soil was prepared for the experiment by drying in the open air, crushing and sifting. The pots were filled with 5 kg of soil each. A week before the plant sowing uniform basic fertilization was applied to each pot: 1 gN as ammonium nitrate, 0.25 gP as sodium dihydrogen phosphate(V) and 1 gK as potassium chloride. Ni was added to the soil as nickel acetate and applied following the experiment design (Table 4).

Ni supplements corresponded to critical values of the subsequent soil pollution degrees with this metal as suggested by The Institute of Soil Science and Plant Cultivation (IUNG) [16]. Basic fertilization and metal supplements were applied as solutions of pure for analysis salts. Spinach, 'Matador' c.v., was cultivated as a test plant.

Table 4

Scheme of pot experiments

Nickel addition to the substratum	
Degree of contamination acc. to criteria of IUNG [16]	[mgNi · kg^{-1}]
Control	Natural content
0^{o}	10
I^{o}	30
II^{o}	50
III^{o}	100

Reduced form of glutathione (GSH) was assessed using the method described by Gurie [17] with modifications. Glutathione was extracted from plant tissues using a mixture of ethylenediaminetetraacetic acid and trichloroacetic acid (EDTA-TCA). In order to bring pH solution to the value of *ca* 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.

In order to assess ascorbic acid the plant material was homogenised with 10 % of oxalic acid solution. After sample centrifugation the supernatant was decanted, its volume was assessed and it was titrated with iodine standard in potassium iodide against 1 % starch solution as an index until intensive blue colour appeared. Ascorbic acid content in the test plants was calculated from the amount of iodine in potassium iodide used for titration [18–20].

Nickel content in spinach aboveground parts was assessed using ICP-AES method after wet mineralization in a mixture of HNO_3 and H_2O_2 (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.

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

Statistical analysis of results was conducted using STATISTICA programme, version 6.0 and Microsoft Excel 2007 calculation sheet. The significance of differences in the analysed chemical compound concentrations in the test plants growing in substrata of various degree of nickel pollution and in successive vegetation phases were assessed using t-Student test at the significance level $\alpha \leq 0.05$.

Results and discussion

Nickel concentrations in spinach growing in the control objects in both experiments ranged from 2.00 to 4.96 mg · kg^{-1} d.m. (Table 5).

Table 5

Ni contents in biomass of spinach in individual phases of plants development [mg · kg^{-1} d.m.]

Phase of plant development	Nickel addition acc. to degree of substratum contamination				
	control	0°*	I°	II°	III°
2010					
I phase	2.10^{a} **	20.60^{a}	66.66^{a}	128.57^{a}	lack of plant material for analyses
II phase	2.00^{a}	24.83^{b}	79.59^{b}	178.64^{b}	
III phase	3.27^{b}	26.21^{b}	77.05^{ab}	168.22^{ab}	
2011					
I phase	4.96^{a}	18.16^{a}	68.94^{a}	204.51^{a}	lack of plant material for analyses
II phase	4.32^{ab}	20.52^{ab}	71.32^{a}	131.48^{b}	
III phase	4.04^{b}	24.28^{b}	82.44^{b}	158.31^{ab}	

* Concerns Tables 5–7 and Figs. 1–3: 0°, I°, II°, III° – Nickel additions corresponded to degree of successive substratum contamination acc. to criteria of IUNG [16]; ** concerns Tables 5–7: different letters indicate significant differences depending on nickel additions in relation to control object and objects with lower degree of pollution, at $\alpha \leq 0.05$.

Applied nickel additions caused a significant increase in this element content in spinach aboveground parts. Chen et al [21] reported that Ni uptake by plants in the first place depends on Ni^{2+} ion concentrations in the soil solution. In the presented research in both years of the experiment the first applied supplement caused a 10-fold increase in Ni accumulation in the aboveground parts, whereas the subsequent two admixtures increased its content about 40 and 60-fold, respectively, in comparison with the control. The largest Ni addition, corresponded to III degree of soil pollution caused an inhibition of spinach growth and development (Fig. 2).

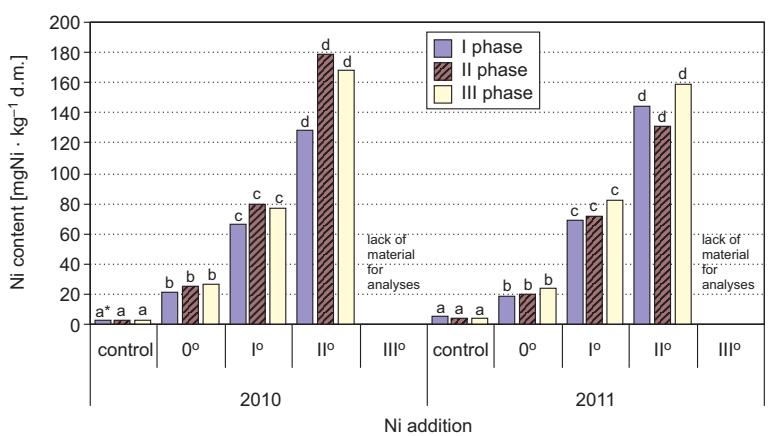

Fig. 2. Ni content in aboveground parts of the spinach in individual phases of development depending on applied metal additions
* Concerns Figs. 2–4: different letters indicate significant differences depending on nickel additions in relation to control object and objects with lower degree of pollution, at $\alpha \leq 0.05$

Kabata-Pendias and Pendias [22] reported that plants growing in the polluted areas may contain even 140 mgNi · kg^{-1} d.m., whereas Molas [23] stated that leaves of cabbage growing in the substratum with between 30 and 90 μmolNi · dm^{-3} contained from 18.21 to 252.54 mgNi · kg^{-1}. Ni contents in spinach aboveground mass harvested from the individual objects in the subsequent development phases generally revealed a growing tendency (Table 5).

Glutathione content in spinach from the objects with Ni addition to the substratum ranged from 41.51 to 140.73 μg · g^{-1} f.m. (Table 6).

Table 6

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

Phase of plant development	Nickel addition acc. to degree of substratum contamination				
	control	0° *	I°	II°	III°
2010					
I phase	78.92[a] **	83.23[a]	91.73[a]	100.21[a]	48.72
II phase	64.47[b]	64.89[ab]	69.87[b]	52.64[b]	—
III phase	31.70[c]	41.51[b]	59.10[c]	73.13[b]	—
2011					
I phase	87.33[a]	103.41[a]	131.04[a]	140.73[a]	80.30
II phase	90.76[a]	111.19[a]	117.47[a]	114.09[b]	—
III phase	59.01[b]	74.36[b]	86.81[b]	108.83[b]	—

Hawrylak and Szymanska [24] in their research on spinach plant response to various selenium forms assessed glutathione content in plants ranging from 34.58 to 51.10 μg · g^{-1} f.m. In the Authors' own investigations, in the first stage of plant growth and for three first applied nickel supplements, corresponded to 0, I and II degree of soil pollution, glutathione content increased proportionately to Ni concentration in the substratum (Fig. 3).

In the objects with nickel supplement of 100 mg · kg^{-1} of soil (corresponded to III degree of pollution) in both years of the experiment spinach contained markedly less of glutathione than plants from the control or from the other objects with lower degree of soil pollution with this element. Srivastava and Dwivedi [25] registered an increase in GSH content in pea plants under the influence of a small addition of salicylic acid to the substratum, whereas bigger amounts of this compound caused a decline in glutathione content. In the first phase of growth the content of reduced glutathione form in spinach from all objects increased proportionately to the Ni amount in soil corresponded to 0, I and II degree of soil pollution. The greatest nickel addition caused a decrease in the content of reduced glutathione in plant biomass. Spinach plants which contained smaller amounts of glutathione than plants from the control or less polluted objects did not tolerate high concentrations of this element in substratum. In result of high toxicity of this metal, plants from the object with high level of substratum pollution with Ni after germination grew poorly and died. Presence of reduced form of glutathione and

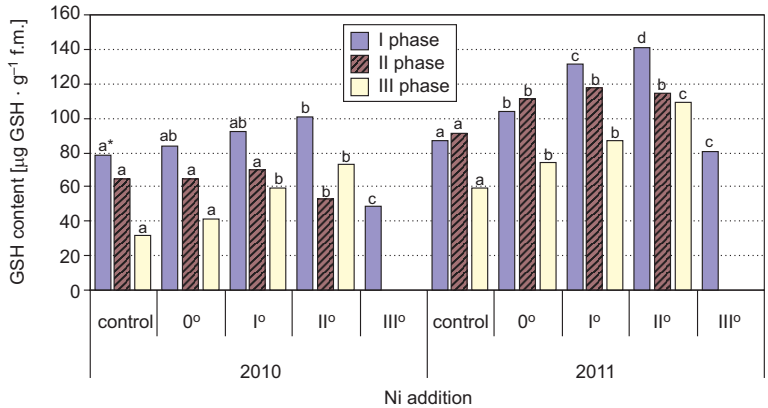

Fig. 3. Glutathione content [µg GSH · g^{-1} f.m.] in aboveground parts of spinach in individual phases of development depending on nickel addition

possibility of regenerating its quota in plant tissue is a condition for tolerance high concentrations of trace elements. Ni present in a plant cell causes numerous disturbances and impairment of all life processes. Each organism has its inherent limit of tolerance to an individual stressor. When the intensity of the stressor is too high and disturbances due to its presence are too serious, the tolerance mechanism fails and the cell dies [26]. Low concentration of reduced glutathione in spinach growing in the substratum with Ni supplement corresponded to III degree of soil pollution might have been connected with high concentration of the element ions in plant cells. Free metal ions, including nickel, impair functions of enzymes, among other glutathione reductase, which is responsible for reduction of oxidized glutathione and therefore for recovering its ability for chelating these ions. Moreover, free ions of toxic elements lead to formation of active oxygen forms which injure and damage lipids, proteins, carbohydrates and nucleic acids, disturbing the correct metabolism of a living cell [27]. Spinach plants growing in the other objects receiving Ni supplement, in the second and third development phase, despite smaller amount of produced biomass than the control plants, tolerated applied additions of this metal. In the 2nd and 3rd phase of growth glutathione content in the plants tolerating this element quantities introduced to the soil and growing in the objects with increasing substratum pollution with Ni, contained greater amounts of GSH. In the 3rd growth phase plants from all objects contained significantly lesser amounts of glutathione in comparison with the analogous objects at earlier stages of growth.

Ascorbic acid (AsA) content in plants from the control in 2010 and 2011 fluctuated from 26.91 to 41.50 mg · 100 g^{-1} f.m. (Table 7).

Kowalska [28] stated that under conditions of diversified liming levels average contents of ascorbic acid in spinach were from 57.62 to 64.36 mg · 100 g^{-1} f.m. On the other hand Ogunlesi et al [29] who assessed ascorbic acid content in spinach by two methods registered between 35.67 and 38.75 mg · 100 g^{-1} f.m. Guri [17] reported mean AsA contents 56.0; 55.4; 56.8 and 55.2 mg · 100 g^{-1} f.m. in plants of four bean

varieties, while after their exposure to ozone ascorbic acid declined to: 51.5; 52.0; 51.8 and 51.6 mg · 100 g^{-1} f.m., respectively.

Table 7

Ascorbic acid content in biomass of spinach in individual phases of plants development [mg AsA · 100 g^{-1} f.m.]

Phase of plant development	Nickel addition acc. to degree of substratum contamination				
	control	0° *	I°	II°	III°
2010					
I phase	41.50[a] **	44.02[a]	43.66[a]	62.05[a]	72.96
II phase	28.89[b]	30.58[b]	35.45[ab]	39.20[b]	—
III phase	26.91[b]	24.13[b]	32.52[b]	33.45[c]	—
2011					
I phase	40.23[a]	35.41[a]	41.31[a]	47.21[a]	73.09
II phase	29.56[b]	29.98[ab]	28.28[b]	46.85[a]	—
III phase	28.16[b]	28.60[b]	26.68[b]	40.39[a]	—

Contents of ascorbic acid in spinach growing in the substrata with nickel additions ranged from 24.13 to 73.09 mg · 100 g^{-1} f.m. (Table 7). The highest contents of this compound were registered in both years of the experiment in the first phase of plant growth and the concentrations increased proportionately to the quantity of Ni added to the soil. Statistically significant increase in ascorbic acid content in plants in comparison with the control was noted when nickel supplement corresponded to the II and III degree of substratum pollution with this element in all three development phases of the test plants in both years of the experiments (Fig. 4).

Considering AsA content in plants during the vegetation period, the Authors in their own studies found, that spinach contained the greatest amounts of this compound in the 1st growth phase, less in the 2nd, whereas in the 3rd phase its contents were markedly

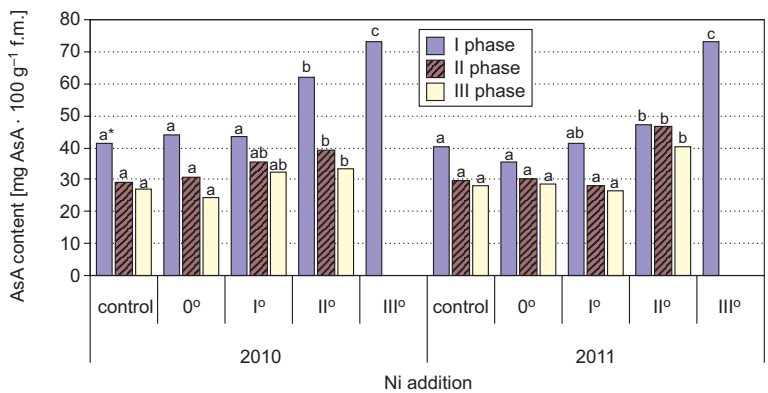

Fig. 4. Ascorbic acid (AsA) content [mg AsA · 100 g^{-1} f.m.] in aboveground parts of spinach in individual phases of development depending on nickel addition

smaller than in the first phase (Table 7). Ascorbic acid content in plants may change under the influence of such factors as: growth intensity, the length of day, the temperature and insolation [30, 31]. Mahan and Wajnura [32] found higher concentrations of ascorbic acid in cotton under conditions of water stress in comparison with plants growing at an adequate irrigation level. Many authors confirm changes in vitamin C content in plants in individual growth phases with a tendency to lowering its level with the plant age [33].

The content of the other investigated cell antioxidant – ascorbic acid in spinach, like glutathione depended on the amount of Ni supplied to the substratum and development phase of the plants. In plants growing in all objects with substrata to various degree contaminated with nickel, increasing content of ascorbic acid was registered along with substratum pollution with this metal. In the objects which received Ni supplement corresponded to the third degree of pollution, concentration of ascorbic acid was the highest, whereas glutathione the lowest in comparison with the control and objects which substratum pollution with this element was lower. A decrease in GSH quota might have been the result of dehydroascorbic acid reduction to ascorbic acid, in which GSH participates. Functions of glutathione and ascorbic acid are interconnected in the Halliwell-Asady cycle [15]. Plants contained the smallest amounts of AsA in the third investigated development phase, which points to better efficiency of antioxidative system in plants in the initial period of their growth and development.

Conclusions

1. Ni doses applied in the experiments caused oxidative stress visible as increased synthesis of antioxidants (GSH and AsA) in spinach plant biomass.

2. Toleration of high Ni concentrations by spinach plants occurs greatly owing to the mechanisms connected with synthesis of antioxidative and buffering compounds (AsA, GSH).

3. During the whole vegetation period and under conditions of stress caused by Ni doses applied to the soil, spinach plants synthetized increased amounts of glutathione, proportionate to Ni content in the soil and plant.

4. Spinach plants growing in Ni contaminated substratum synthetized significantly bigger quantities of ascorbic acid than the control plants in each development phase and in both years of the experiments.

5. The antioxidative system in spinach plant cells acts more efficiently at the initial stage of vegetation, as evidenced by much higher contents of glutathione and ascorbic acid in the first phase of this plant development. At the subsequent stages of plant development successive decrease in antioxidative compounds (GSA and AsA) were observed.

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SPRAWNOŚĆ SYSTEMU ANTYOKSYDACYJNEGO ROŚLIN SZPINAKU ROSNĄCYCH W GLEBIE ZANIECZYSZCZONEJ NIKLEM

Katedra Chemii Rolnej i Środowiskowej
Uniwersytet Rolniczy im. Hugona Kołłątaja w Krakowie

Abstrakt: W pracy podjęto próbę oceny aktywności systemu antyoksydacyjnego komórek roślin szpinaku odmiany ‘Matador’ rosnących w glebie zanieczyszczonej Ni. Materiał roślinny do analiz pozyskano w dwóch doświadczeniach wazonowych prowadzonych w latach 2010 i 2011 w hali wegetacyjnej stacji doświadczanej

Uniwersytetu Rolniczego w Krakowie. W częściach nadziemnych roślin oznaczono zawartość Ni metodą ICP-ES, zredukowanej formy glutationu metodą kolorymetryczną oraz kwasu askorbinowego metodą miareczkową.

Zawartość niklu w częściach nadziemnych szpinaku wynosiła od 2,00 do 204,5 mg · kg^{-1} s.m. i zwiększała się wraz ze stopniem zanieczyszczenia podłoża tym pierwiastkiem oraz na ogół wraz z wiekiem roślin. Rośliny zawierały od 31 do 238 μg GSH · g^{-1} ś.m. W roślinach z pierwszych trzech obiektów o zanieczyszczeniu podłoża odpowiadającym 0^o, I^o, II^o, według klasyfikacji IUNG, zawartość tego antyoksydantu była większa w porównaniu z zawartością GSH w roślinach z obiektu kontrolnego i obiektów o niższym stopniu zanieczyszczenia. W obiekcie z największym zastosowanym dodatkiem niklu zawartość GSH w roślinach zmniejszyła się istotnie w porównaniu z roślinami z pozostałych obiektów, a rośliny z tego obiektu obumarły niedługo po wschodach. Zawartość kwasu askorbinowego w szpinaku w obydwu latach doświadczeń mieściła się w przedziale od 24,13 do 73,09 mg · 100 · g^{-1} ś.m. i zwiększała się w roślinach z kolejnych obiektów o coraz większym zanieczyszczeniu podłoża niklem.

Rośliny szpinaku w pierwszej fazie wzrostu zawierały na ogół znacznie więcej GSH i AsA, co wskazuje na znacznie wyższą sprawność systemu antyoksydacyjnego roślin w początkowym okresie wzrostu.

Słowa kluczowe: system antyoksydacyjny, glutation, kwas askorbinowy, nikiel, szpinak