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Nuclear Microprobe Studies of Grasshopper Feeding on Nickel Hyperaccumulating Plants

M. Augustyniak[(a)], W. Przybyłowicz[(b,c)], J. Mesjasz-Przybyłowicz[(b)], M. Tarnawska[(a)], P. Migula[(a)], E. Głowacka[(d)], A. Babczyńska[(a)]

[(a)] *Department of Animal Physiology and Ecotoxicology, University of Silesia, Bankowa 9, 40-007 Katowice, Poland*
[(b)] *Materials Research Group, iThemba LABS, PO Box 722, Somerset West 7129, South Africa*
[(c)] *Faculty of Physics and Applied Computer Science, AGH University of Science and Technology, Kraków, Poland*
[(d)] *Department of Zoology, University of Silesia, Bankowa 9, 40-007 Katowice, Poland*

Abstract. Grasshopper *Stenoscepa* sp. is an insect species feeding on the South African Ni-hyperaccumulating plants. Large amounts of Ni ingested by them have no effect on their development. In order to explain the abilities to survive in such extreme conditions we investigated the distribution of elements in the insect body by means of a nuclear microprobe (micro-PIXE and BS). GeoPIXE II software was used for quantitative elemental mapping complemented by evaluation of data extracted from arbitrarily selected micro-areas. Micro-PIXE analysis in *Stenoscepa* sp. tissues showed the highest Ni level in the gut and Malpighian tubules. The activity of glutathione-dependent enzymes and glutathione (GSH) content in the tissues of 2nd stage larvae were measured. One of the ways to survive under chronic Ni exposure conditions is an intensified GSH synthesis. GSH concentration in tissues of the grasshoppers was very high, about six times higher than in larvae of other Acrididae species from areas contaminated with heavy metals in Europe. Catalase activity was 5-10 times lower in comparison to other Orthoptera species. Glutathione reductase (GR) activity was unexpectedly low (at the detection limit level). Likely the studied grasshoppers may use other metabolic pathways for regeneration of the reduced form of GSH, e.g. thioredoxine system.

Keywords: nickel, *Stenoscepa* sp., micro-PIXE, X-ray microanalysis, glutathione, GST, GPx, GR, CAT

INTRODUCTION

Hyperacumulating plants, capable to accumulate extremely high metal concentrations, are regarded as very promising in phytoremediation and/or phytomining (Brooks et al., 1998). This extraordinary feature of hyperacumulating plants is a challenge for rare phytophagous insects evolutionarily adapted to live and develop in such extreme conditions. The discovery of specific adaptation mechanisms of certain animals to high metal concentrations gives a possibility of studies leading to understanding the phenomena taking place in industrially affected ecosystems, quite numerous worldwide.

The grasshopper *Stenoscepa* sp. is one of a few insect species feeding on hyperaccumulating plants (Mesjasz-Przybyłowicz and Przybyłowicz, 2001). Our test of choice experiments, carried on the 2nd stage larvae, demonstrated that this insect prefers 3 out of 7 offered plants. These three plants - *Berkheya coddii, Berkheya zeyheri* and *Senecio coronatus* - are Ni-hyperaccumulators, growing on ultramafic soils containing high concentrations of Fe, Cr and Ni. They belong to the group of five Ni-hyperaccumulators found in South Africa (Smith et al., 2001). For example, *B. coddii* can store up to 76,100 mg $Ni \cdot kg^{-1}$ dry weight of leaves (Mesjasz-Przybylowicz et al., 2004). We know that *Stenoscepa* sp. can feed exclusively on these plants and, moreover, large amounts of nickel in leaves are not toxic for them. This particular ability stimulated us to search for symptoms of the adaptation to survive in such extreme conditions. In this study we measured the contents and distribution of nickel and other elements in tissues and organs of *Stenoscepa* sp.

It is known that metals (including Ni) might have an indirect effect on the activity of detoxifying enzymes, as well as on the concentration of metal-binding peptides. Among the peptides glutathione plays a significant role. This tripeptide is necessary in many detoxifying reactions. It is crucial for proper functions of glutathione peroxidase (GPx), glutathione transferase (GST), glutathione reductase (GR) and many other enzymes. Moreover, due to its structure, glutathione itself can bind metals, diminishing their toxicity. Earlier study conducted on grasshoppers from meadows located along a heavy metal pollution gradient showed that in specimens from the most polluted site, the level of glutathione was lower than in individuals from less contaminated areas (Augustyniak and Migula 2000). We supposed that glutathione could be partly used for eliminating excessive amounts of metals. Glutathione-metal complexes cannot be reused in other metabolic pathways. The data, however, refer to insects exposed to high concentrations of metals of industrial origin. In such conditions the exposure may last several tens or hundreds years. In case of *Stenoscepa* sp. we suppose that time needed for adaptation of the insects to the diet naturally enriched with nickel was significantly longer than for insects living under industrial stress. Thus, in this study we measured glutathione concentration and the activity of GSH-dependent enzymes in the 2nd stage larvae tissues, to understand adaptation mechanisms in insects living in habitats heavily polluted with metals.

MATERIALS AND METHODS

The 2nd larval stage of *Stenoscepa* sp. (Orthoptera) was used. Like other grasshoppers, the insects are very fertile, able to inhabit various niches, including heavily polluted environments. The insects were collected in early spring on sprouts of *B. coddii* that cover ultramafic valley at Agnes Mine (Mpumalanga Province, South Africa).

Elemental microanalyses using PIXE

After catching grasshoppers were anaesthetized on ice and selected parts of the body were dissected. The tissues were immediately cryofixed in liquid propane cooled by liquid nitrogen (using Leica CPC) and then freeze-dried for 24 hours (using Leica CFD). Samples were then mounted between two layers of Formvar film with front side coated with carbon. Micro-PIXE measurements were performed using a nuclear microprobe (Przybyłowicz et al., 2004). A proton beam of 3.0 MeV energy and 200-300 pA current was focused to 3 μm × 3 μm spot and raster scanned over grasshoppers' tissues. External absorber (190 μm Kapton foil) was positioned between the PIXE Si(Li) detector and the specimen. Data were collected using XSYS data acquisition system. Further analysis was performed using GeoPIXE II software package (Ryan, 2000). The matrix composition and area density was obtained from analysis of corresponding proton backscattering (BS) spectra using a RUMP simulation package (Doolittle, 1986). Generation of true, quantitative elemental maps was performed using Dynamic Analysis method. Metal concentrations in each sample were expressed in $\mu g \cdot g^{-1}$ dry weight.

Enzyme activity assays

The 2nd instar of *Stenoscepa* sp. were anesthetized on ice and next individually homogenized in 0.05 M phosphate buffer (pH 7.4) at 4°C. The homogenates were centrifuged for ten minutes at 15,000 *g* at 4°C. Enzyme activities as well as glutathione content were measured in the submitochondrial fraction. Total glutathione content was measured using the method of Griffith (1980). Glutathione S-transferase (GST) [EC 2.5.1.18] activity was determined using the method of Yu (1982). Glutathione reductase (GR) [EC 1.6.4.2] activity was measured using the method described by Racker (1955). Se-independent glutathione peroxidase (GPx) [EC 1.11.1.9] activity was assayed as in Simmons et al. (1989). Catalase (CAT) [EC 1.11.1.6] activity was assayed using the method of Aebi (1984). Protein concentration was determined using the method described by Bradford (1976).

RESULTS AND DISCUSSION

Protection of crucial organs and tissues against harmful effects of metals is a well recognized mechanism of tolerance of extremely high metal concentrations in food and environment. This was found in insects exposed to metals of both natural and anthropogenic origin. For example, in grasshoppers living on polluted meadows in southern Poland the brain and gonads contained less amount of metals than other organs (Augustyniak and Migula, 2000; Augustyniak et al., 2006). *Stenoscepa* sp. is one among a few insect species that live on *Berkheya coddii*. The best recognized and studied is a monophagous beetle *Chrysolina pardalina* (Mesjasz-Przybylowicz and Przybylowicz, 2001; Augustyniak et al., 2002; Mesjasz-Przybylowicz et al., 2002; Przybyłowicz et al., 2003, 2004). These insects accumulate Ni mainly in the Malpighian tubules (up to 620 $\mu g \cdot g^{-1}$), and in the midgut. The excess of metal is rejected from the body mainly with faeces (Przybyłowicz et al., 2003, 2004). Micro-PIXE analyses showed that reproductive organs or neuronal structures are efficiently protected against the excess of nickel (only 6.9 μg $Ni \cdot g^{-1}$). In sap-feeding insects, such as *Protaphis pseudocardui* (Aphidinae) and *Norialsus berkheyae* (Cixiidae, Hemiptera),

TABLE 1. Concentration of selected elements ($\mu g \cdot g^{-1}$) with 99% detection limits (in brackets) in brain, gut, pyloric valves and Malpighian tubules of 2nd instar of *Stenoscepa* sp. Results based on PIXE spectra extracted from respective regions.

Tissue/organs	Ni	Ca	Fe	Zn	Cu	S
Brain	85.6 (0.4)	155 (1.7)	64.8 (0.3)	72.0 (0.36)	9.6 (0.37)	3461 (119)
Gut	697 (0.7)	1790 (3.2)	48.2 (0.5)	54 (0.71)	9.1 (0.78)	3125 (203)
Pyloric valves	435 (1.3)	181 (4.2)	140 (0.95)	35 (1.3)	6.3 (1.5)	3231 (247)
Malpighian tubules	758 (8.8)	296 (14)	118 (6.9)	40 (6.2)	45 (8.5)	816 (44)

Ni concentration in the head was lower than in their thorax and abdomen (Migula et al., 2005). In the thrips *Haplothrips acanthoscelis* feeding on *B. zeyheri* Ni concentration was only 1.4 $\mu g \cdot g^{-1}$ in the head (Przybyłowicz et al., 2004). However, this protection hypothesis does not refer to all insect species. For example, Ni concentration in the head of *Orthesia* sp. (Pseudococidea - a root-dweller of *B. coddii*) was 110 μg Ni$\cdot g^{-1}$, twice as high as in the whole body (Migula et al., 2005). Ni concentrations in various tissues and organs of *Stenoscepa* sp. are the highest among all insect species feeding on South African Ni-hyperaccumulators. Analysis of concentration and distribution of Ni in *Stenoscepa* sp. tissues performed by micro-PIXE showed the highest Ni level in the gut (in the peritrophic membrane areas and in the pyloric valves - over 1000 $\mu g \cdot g^{-1}$). In Malpighian tubules Ni content was above 700 $\mu g\ g^{-1}$, while in the brain the mean concentration value was 85.6 $\mu g \cdot g^{-1}$ (Table 1).
The analysis of Ni distribution in the brain of *Stenoscepa* sp. reveals further facts (Fig. 1): Ni concentration in optic lobes (31 $\mu g \cdot g^{-1}$) was lower than in the cerebral ganglia (246 $\mu g \cdot g^{-1}$) (Fig. 1, brain: region Q1 vs. region Q2). Ni concentration was also measured in the suspension of neurons isolated from *Stenoscepa* sp. brain. We found that Ni concentration in the neurons reached 1000 $\mu g \cdot g^{-1}$ (Fig. 2). Summarizing, the majority of toxic Ni is retained in the alimentary tract of *Stenoscepa* sp. This metal migrates to gut epithelial cells that are peeled off and removed from the organism. Still, a large amount of nickel enters the organism of the grasshopper. It is known that nickel is a transition metal that often takes part in oxidation-reduction processes. It may cause an increase in free radical concentration in an organism (Ahmad, 1995). The increase is reflected by the activity rates of catalase, glutathione peroxidase and glutathione reductase. Moreover, Ni can bind to glutathione, thus in animals exposed to this metal the concentration of this tripeptide as well as the activity of glutathione-dependent enzymes might change. Tissue level of GSH in *Stenoscepa* sp. was very high (average value 29.59 $\mu g \cdot mg^{-1}$ protein). In comparison to larvae of other Acridid species inhabiting areas contaminated with heavy metals in Europe this amount was about six times higher. GSH-dependent enzymes' activity was at similar or much lower level as in other Orthoptera larvae, which also grow in environment contaminated with heavy metals.

TABLE 2. Glutathione contents (GSH/GSSG) and activity of glutathione S-transferase (GST), Se-independent glutathione peroxidase (GPx), glutathine reductase (GR) and catalase (CAT) in the 2nd instar of grasshopper – *Stenoscepa* sp. (mean±SD; n=7).

Parameter	Value	Units
GSH	29.59±5.25	[$\mu g \cdot mg$ protein^{-1}]
GST	51.86±11.06	[nmol$\cdot min^{-1} \cdot mg$ protein^{-1}]
GPx	43.35±9.91	[nmol$\cdot min^{-1} \cdot mg$ protein^{-1}]
GR	0.61±0.28	[nmol$\cdot min^{-1} \cdot mg$ protein^{-1}]
CAT	4.87±1.05	[μmol$\cdot min^{-1} \cdot mg$ protein^{-1}]

Catalase activity was also 5-10 times lower in comparison to other Orthoptera and about 100 times lower than in *C. pardalina* (Augustyniak et al., 2006; 2007), while glutathione reductase (GR) activity in *Stenoscepa* sp. body was unexpectedly low (at the detection limit level). This result is surprising, since it indicates insignificant role of the regeneration of reduced form of glutathione. Opposite effects were

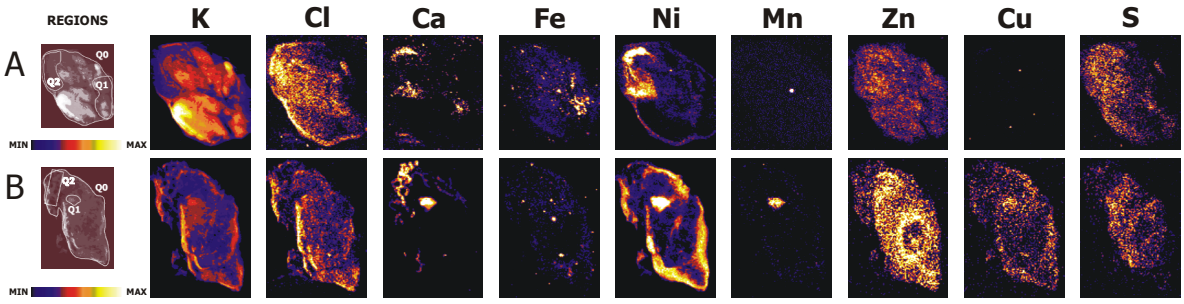

FIGURE 1. True elemental maps of *Stenoscepa* sp.: (A) brain Q0 (Q1 - optic lobes; Q2 - cerebral ganglia) and (B) gut Q0 (Q1 - inorganic concretions supposedly intaken with food by grasshopper; Q2 –fragment with Malpighian tubules).

found in *C. pardalina*, where GR activity was significantly higher, what suggests that regeneration of reduced glutathione is important in this species. Also, the activity of glutathione-dependent enzymes was higher in *C. pardalina* than in *Stenoscepa* sp. It is worth stressing that glutathione concentration in *C. pardalina* was 20-50 times lower than in *Stenoscepa* sp. (Augustyniak et al., 2007).

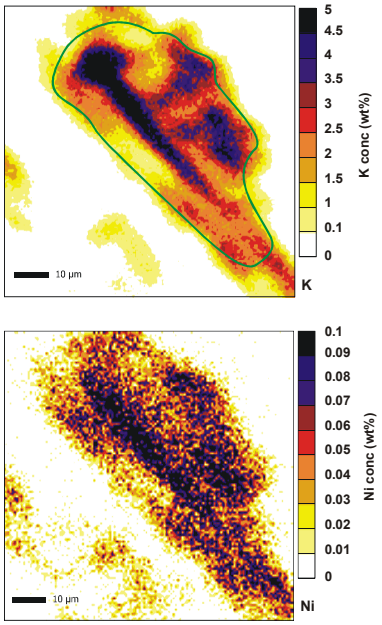

FIGURE 2. True elemental maps (K and Ni) of the neurons isolated from the brain of the 2nd instar of *Stenoscepa* sp.

Concluding, we may say that the adaptation of *Stenoscepa* sp. to high nickel concentration in food is based on enhanced excretion of this element with faeces. In this process Malpighian tubules, which are well developed in this grasshopper, play an important role. However, the gut barrier is likely not as efficient as in *C. pardalina* and relatively large amounts of nickel migrate to the haemolymph and other tissues. Intensive glutathione synthesis seems to be another adaptation to high nickel concentration. The tripeptide is used mainly for binding nickel. Glutathione-dependent biochemical pathways are of minor importance in this species.

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