Impact of Zinc on Heavy Metals Content, Polyphenols and Antioxidant Capacity of Faba Bean in Milk Ripeness

M. Timoracká, A. Vollmannová., D.S. Ismael, J. Musilová

Abstract**—**We investigated the effect of targeted contaminated soil by Zn model conditions. The soil used in the pot trial were uncontaminated. Faba beans (cvs Saturn, Zobor) were harvested in milk ripeness. With increased doses applied into the soil the strong statistical relationship between soil Zn content and Zn amount in seeds of both of faba bean cultivars was confirmed. Despite of high Zn doses applied into the soil in model conditions, in all variants the determined Zn amount in faba bean cv. Saturn was just below the maximal allowed content in foodstuffs given by the legislative. In cv. Zobor the determined Zn content was higher than maximal allowed amount (by 2% and 12%, respectively). Faba bean cvs. Saturn and Zobor accumulated (in all variants higher than hygienic limits) high amounts of Pb and Cd. The contents of all other heavy metals were lower than hygienic limits. With increased Zn doses applied into the soil the total polyphenols contents as well as the total antioxidant capacity determined in seeds of both cultivars Saturn and Zobor were increased. The strong statistical relationship between soil Zn content and the total polyphenols contents as well as the total antioxidant capacity in seeds of faba bean cultivars was confirmed.

Keywords**—**antioxidant capacity, faba bean, polyphenols, zinc

INTRODUCTION

Agricultural production is the main source of foodstuffs, it is important to evaluate negative effects of risky elements on quality of agricultural products. The monitoring of heavy metals content is very important because consumption of legume is necessary for human nutrition. Soil is a dynamic system which is influenced by various factors, whether natural or anthropic, causing the contamination. Changes that occur due to these factors in the soil cause bioaccessibility of metals and can enrich the soil with other elements that are biologically active, or contrary, degrade a land, and it becomes inappropriate for crop growth. Heavy metals occur naturally in the ecosystem with large variations in concentration. Living organisms require varying amounts of "heavy metals." Iron, cobalt, copper, manganese, molybdenum, and zinc are required by humans. Excessive levels can be harmful to the organism. Certain elements that are normally toxic, for certain organisms or under certain conditions, beneficial. Some of these elements are actually necessary for humans in trace amounts (cobalt, copper, chromium, manganese, nickel) while others are carcinogenic or toxic, affecting, among others, the central nervous system (manganese, mercury, lead, arsenic), the kidneys or liver (mercury, lead, cadmium, copper) or skin, bones, or teeth (nickel, cadmium, copper, chromium) [1]. Plants which exhibit hyperaccumulation can be used to remove heavy metals from soils by concentrating them in their biomatter. Legumes are rich and inexpensive source of nutrietns to millions of peoples but also an excellent Zn accumulator [2]. Zn as well as Cu are essential micronutrients, they can be toxic when taken in excess. Lead and cadmium are nonessential metals as they are toxic, even in trace (Gençcelep et al. 2009). Relations between toxic metals (Pb, Cd) and essential elements (eg. Zn) are very important for mineral balance, because heavy metals can cause a lack of some essential elements in plants. Next, the presence of antinutrient, e.g. polyphenols seems to be one of the reasons why zinc is a limiting nutrient in many diets of people. On the other hand, polyphenols presence in food is connected with antioxidant effect on human health.

Therephore we investigated the effect of accumulation of heavy metals in faba bean grown in model conditions in the targeted contaminated soil with increasing rates of the selected heavy metal (zinc). Zinc was added to the soil to reduce the intake of other heavy metals, especially of Cd or Pb (these elements are present in the soil above the hygienic limit on most territory of the Slovak Republic) as well as to determine the level of benficial and safety intake of observed elements on human organism. Neither, we also investigated the effect of accumulation of selected micronutrient content (zinc) on total

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polyphenols and antioxidant activity assessed in faba bean grown in soil with increasing rates of the zinc doses.

MATERIALS AND METHODS

In the pot experiments the soil from locality Cakajovce was used. Slovak village Cakajovce is located in Nitra upland with annual rainfall 600-700 mm and annual temperature 8 - 9º C. Before the realization of pot experiments the soil from this locality was sampled by valid method with pedological probe GeoSampler fy. Fisher and processed as described above.

The cultivars of faba bean (Saturn, Zobor) used in the pot experiments were obtained from the Research Centre of Plant Production in Piestany (Slovakia). Faba beans were harvested in milk ripeness.

In model conditions of the vegetation pot experiments realised at the Department of Chemistry, we investigated the effect of addition of independent increasing rates of selected micronutrient (zinc) into the soil and its consecutive accumulation and polyphenols production in faba bean seeds.

Zinc was applied in the form of $ZnSO_4.7H_2O$.

For cvs. Zobor and Saturn, the experiments were realised:

A: control (without Zn addition)

B: 40 mg $Zn.kg^{-1}$ of soil (hygienic limit)

C: 250 mg $Zn.kg^{-1}$ of soil (half dose of analytically significant contamination)

D: 500 mg $Zn.kg^{-1}$ of soil (analytically significant contamination)

The experiment was based on four replications in each variant.

Chemical analysis of the soil. Analyses were conducted in samples of soil ground on fine soil I. and from this fine soil the representative sample was taken and sieved through the sieve with average 0.2 mm (fine soil II). In each soil sample the exchangeable reaction (pH/KCl), the contents of available nutrients (K, Mg, P) and mobile forms of Ca according Mehlich II. and content of humus by Tjurin method were determined. Pseudototal content of risk metals including all of the forms besides residual metal fraction was assessed in soil extract by aqua regia and content of mobile forms of selected heavy metals in soil extract by NH_4NO_3 (c=1 $mol.dm^{-3}$) and HNO_3 (c=2 $mol.dm^{-3}$). Gained results were evaluated according to Law No. 220/2004 - extract by NH_4NO_3 and Decision of Ministry of Agriculture in Slovak republic about highest acceptable limits of toxic compounds in soil No. 531/1994 – 540 - extract by HNO_3 (valid in the Slovak Republic) as well as threshold values proposed by European Commission (EC) (2006). Analytical ending was flame AAS (AAS Varian AA Spectr DUO 240 FS/240Z/UltrAA).

Heavy metals in the plant material. The samples of legume seeds were collected from the same sampling points as the soil samples. After their dryining and regulation the plant samples were decomposited with using of HNO_3 in the microwave digestion instrument MARS X-PRESS. The solutions were analyzed by flame AAS (AAS Varian AA Spectr DUO 240 FS/240Z/UltrAA). Gained results in $mg.kg^{-1}$ of fresh mater (FM) were evaluated according to the Food Codex of the Slovakia valid in the Slovak Republic (FC SR) as well as according to Commission Regulation 1881/2006 (CR). Maximum levels for the content of risky metals in foodstuffs in these legislative norms are given in $mg.kg^{-1}$.

Phenolics extraction. Phenolic compounds were extracted from seeds by preparation of methanol extracts. Methanol is a typical solvent for the extraction of phenolic acids and flavonoids. For 12 hours extraction, dry milled material (10 g) was used and continuously extracted by a Twisselmann extractor with methanol (80%, v/v).

Total polyphenol content determination (TP). The amount of total phenolics was determined using Folin-Ciocalteau reagent (FCR) (Merck, Germany) according to [3]. Sample extracted (0.05 g to 1mL of 80% methanol according to the expected polyphenol content), 2.5 mL of FCR and 3 mL of H_2O were added to a 50 mL flask. After 3 minutes 7.5 mL of Na_2CO_3 (20%) were also added to the flask and diluted to 50 mL with H_2O. The mixture was then incubated for two 2h at laboratory temperature and the absorbance was measured at 765 nm on a Shimadzu spectrophotometer (710, Shimadzu, Kyoto, Japan) against a blank (sample extract replaced with 80% methanol). The amount of total phenolics was calculated as gallic acid equivalents (GAE) in milligrams per kilogram fresh sample.

Total antioxidant capacity determination (TAC). For the analysis of free radical scavenging activity 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used according to the protocol in [4]. To obtain a stock solution: 0,025 g of DPPH (Sigma-Aldrich) was diluted to 100 mL with methanol (Spectranal Ridel de Haen, Hanover, Germany), and kept in a cool and dark place. Immediately before the analysis, a 1:10 dilution of the stock was made with methanol. For the analysis, 3.9 mL of the DPPH working solution was added to a cuvette and the absorbance at 515 nm was measured (A0) with a Shimadzu spectrophotometer (710, Shimadzu, Kyoto, Japan). Subsequently, 0.1 mL of the extract was added to the cuvette with DPPH, and the absorbance was measured after 10 min (A10). An increasing amount of antioxidants present in the methanol extract of the sample reduced DPPH inhibition according to the following equation:

$$\text{Inhibition (\%)} = [(At0-At10)/At0] \times 100 \qquad (1)$$

RESULTS AND DISCUSSION

Two experiments were realised as the pot trials in the vegetation cage of the Department of Chemistry of Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra with the aims to investigate the relationship between soil content of chosen risky metals and their accumulation in seeds of faba bean. In the pot trials the soils from the locality Cakajovce was used.

The soil evaluation. The soil from Cakajovce locality is characterized by low supply of humus and the neutral soil reaction suitable for the legume cultivation. The used soil is characterized also by high content of potassium and phosphorus as well as by a very high content of magnesium (Table I).

TABLE I
AGROCHEMICAL CHARACTERISTICS, MACROELEMENTS CONTENT ($MG.KG^{-1}$) IN THE SOIL FROM LOCALITY CAKAJOVCE (SLOVAKIA)

Agrochemical characteristics	pH (H2O)	pH (KCl)	Cox (%)	humus (%)	
	8.53	7.23	1.53	1.44	
Macroelements	N	K	Ca	Mg	P
	1225	291	5210	380	90.8

The soil used in the pot trial was uncontaminated. Only determined Cd content was on the level of limit value given by Law No. 220/2004 for the soil extract by aqua regia as well as Pb content on the level of critical value given by Law No. 220/2004 for the relationship between soil and plant. The values were far below threshold values proposed by EC (Table II).

TABLE II
HEAVY METAL CONTENTS ($MG.KG^{-1}$) IN THE SOIL FROM LOCALITY CAKAJOVCE (SLOVAKIA)

Heavy metals	Cu	Zn	Co	Cd	Pb	Cr	Ni
Aqua regia	19.8	48.5	13.0	0.72	18.3	27.4	29.2
limit value*	**60**	**150**	**15**	**0.7**	**70**	**70**	**50**
threshold value***	**100**	**200**	-	**1.5**	**100**	**100**	**70**
HNO_3	8.3		5.5	0.29	9.6	1.8	7.3
reference value**	**20**	**40**	-	**0,3**	**30**	**10**	**10**
NH_4NO_3	0.085	0.025	0.14	0.026	0.11	0.06	0.16
critical value*	**1.0**	**2.0**	-	**0.1**	**0.1**	-	**1.5**

*Law No. 220/2004

** Decision No. 531/1994 – 540

***European Commission (2006)

The evaluation of application of graded Zn doses into soil. Seeds of faba bean harvested of milk ripeness are consumed in Slovakia, so the determined contents of heavy metals were compared with limit values given by Food Codex of the Slovak Republic valid in the Slovak Republic (FC SR) as well as according to Commission Regulation 1881/2006 (CR).

Fresh faba bean seeds can be used as vegetable into various salads. In fresh seeds of cv. Saturn (Table III) in control variant the extremely high content of Pb (by 310% higher than maximal allowed amount given by the legislative) was determined. In all variants the determined Pb content was increased and the highest Pb content was determined in D variant (by 500% higher than limit value). Also Cd content in fresh seeds of faba bean Saturn was increased in variants with application of 250 and 500 mg $Zn.kg^{-1}$, but the highest Cd content in D variant was still below the limit value. Contents of other observed metals were lower in variants with Zn application in relation to control variant (with exception of Zn) and were far below the limits.

TABLE III
HEAVY METALS CONTENTS ($\overline{X}$ ± S.D.) IN THE FABA BEAN SATURN ($MG.KG^{-1}$)

Variant	Zn	Cu	Ni	Cr	Pb	Cd
control	8.46±0.02	2.25±0.03	0.87±0.07	0.40±0.01	0.83±0.02	0.03±0.01
40 mg $Zn.kg^{-1}$	9.42±0.11	1.83±0.02	0.42±0.02	0.40±0.03	0.97±0.05	0.02±0.01
250 mg $Zn.kg^{-1}$	14.84±0.25	1.79±0.03	0.63±0.01	0.24±0.03	1.00±0.03	0.04±0.01
500 mg $Zn.kg^{-1}$	17.70±0.08	2.26±0.08	0.73±0.02	0.37±0.02	1.20±0.08	0.06±0.01
Limit*	**50.0**	**15.0**	**3.0**	**4.0**	**0.2**	**0.1**
Maximal level**	-	-	-	-	**0.2**	**0.1**

*Limit value for legumes according to the Food Codex of the Slovakia

**Maximal level according to Commission Regulation 1881/2006

In fresh seeds of faba bean cv. Zobor (Table IV) in control variant the extremely high contents of Pb and Cd (by 630% and 30% respectively higher than maximal allowed amounts given by the legislative) were determined. In B, C and D variants with graded Zn doses the determined Pb contents were by 685%, 545% and 610% higher than limit value (respectively). The determined Cd content was in B and C variants identical (by 60% higher than hygienic limit) and in D variant with the highest Zn dose applied into the soil the determined Cd content was by 30% lower than maximal allowed amount in foodstuffs. The determined Zn content was in variants with Zn application increased, but even in D variant it was lower than the hygienic limit. Contents of Cu, Ni and Cr were only slightly changed in variants with Zn application, only Ni content determined in D variant was 2.4 fold lower than that in the control variant.

TABLE IV
HEAVY METALS CONTENTS ($\overline{X}$ ± S.D.) IN THE FABA BEAN ZOBOR ($MG.KG^{-1}$)

Variant	Zn	Cu	Ni	Cr	Pb	Cd
control	9.02±0.07	1.89±0.01	1.15±0.02	0.42±0.03	1.46±0.03	0.13±0.01
40 mg $Zn.kg^{-1}$	12.33±0.02	1.90±0.02	1.17±0.04	0.34±0.01	1.57±0.10	0.16±0.01
250 mg $Zn.kg^{-1}$	15.58±0.05	1.93±0.07	1.13±0.04	0.42±0.03	1.29±0.02	0.16±0.01
500 mg $Zn.kg^{-1}$	20.37±0.09	1.96±0.02	0,47±0.02	0.42±0.02	1.42±0.18	0.07±0.01
Limit*	**50.0**	**15.0**	**3.0**	**4.0**	**0.2**	**0.1**
Maximal level**	-	-	-	-	**0.2**	**0.1**

*Limit value for legumes according to the Food Codex of the Slovakia

**Maximal level according to Commission Regulation 1881/2006

The determined contents of Cr, Cu and Pb (0.1 mg.kg^{-1}, 0.7 mg.kg^{-1} and 0.1 mg.kg^{-1}, respectively) by [5] in faba bean seeds were many times lower than those determined in our faba bean cultivars, only Ni content determined by these authors was similar to that in our samples (3.4 mg.kg^{-1}). On other hand, reference [6] determined higher amounts of Cr, Cu (11.25 mg.kg^{-1} and 18 mg.kg^{-1}, respectively), a lower Pb content (1.5 mg.kg^{-1}) and a similar Ni content (3.83 mg.kg^{-1}) in comparison to our results.

The graded Zn doses applied into the soil in the model conditions resulted in increased Zn content in seeds of faba bean harvested in the stage of milk ripeness. The strong statistical relationship between soil Zn content and Zn amount in seeds of both of investigated faba bean cultivars was confirmed (R=0.944 and R=0.965, respectively). Despite of very high Zn doses applied into the soil, the determined Zn amount in seeds of both of faba bean cultivars was lower than maximal allowed content in foodstuffs given by the legislative.

References [7]-[8] postulated that some metals such as Zn, Cu, Ni and Cr are essential or beneficial micronutrients for plants, animals and microorganisms, whereas others, such as Cd, Hg, and Pb have no known biological and/ or physiological functions. However, all these metals could be toxic at relative low concentrations. These metals are taken up from soils and bioaccumulated in crops, causing damage to plants when reach high levels and under certain conditions becoming toxic to human and animals feed on these metal enriched plants [9]. Heavy metal accumulation in plants depends upon plant species, and effeciency of different plants in absorbing metals in evaluated by either plant uptake or soil to plant transfer factors of the metals [10].

The total polyphenols and total antioxidant capacity evaluation. Total polyphenol content (TP) and total antioxidant capacity (TAC) determined in seeds of both of investigated faba bean cultivars harvested in the stage of milk ripeness are presented in Table V. The determined values of total polyphenol content were in interval 2208 – 4622 mg GAE.kg^{-1} FM (after calculation to dry mater 10045 – 23225 mg GAE.kg^{-1} DM). Generally, in all variants the determined TP values in seeds of faba bean cv. Zobor were lower in comparison to cv. Saturn with exception of D variant. The determined values of total antioxidant capacity (TAC) were in interval 3.23 – 5.71% DPPH and in all variants the TAC values determined in seeds of faba bean cv. Zobor were higher than those in cv. Saturn. Reference [11] determined total content of polyphenols and antioxidant capacity of thirteen genotypes of faba bean (16980 – 67470 mg GAE.kg^{-1} DM; 2.15 – 28.60% DPPH). These results correspond with our findings.

TABLE V
TOTAL POLYPHENOL CONTENT (MG GAE.KG^{-1}) AND TOTAL ANTIOXIDANT CAPACITY (% OF DPPH INHIBITION)

Variant	Total polyphenol content		Total antioxidant capacity	
	Saturn	Zobor	Saturn	Zobor
control	2208±10	2360±11	3.23±0.02	4.66±0.06
40 mg Zn.kg^{-1}	4087±12	2758±16	3.58±0.01	4.80±0.01
250 mg Zn.kg^{-1}	4622±21	2530±19	3.52±0.01	4.80±0.02
500 mg Zn.kg^{-1}	2987±11	4095±12	4.52±0.01	5.71±0.01

With increased Zn doses applied into the soil in the model conditions the TP contents determined in seeds of both of faba bean cultivars Saturn and Zobor harvested in the stage of milk ripeness were increased. The maximal TP content in seeds of faba bean cv. Saturn can be expected at 200 – 300 mg Zn applied into 1 kg of the soil (Fig. 1), while after application of higher Zn doses into the soil a lower TP content in seeds of cv. Saturn can be expected. The strong statistical relationship between soil Zn content and TP amount in seeds of this faba bean cultivar was confirmed (R= 0.875). On other hand, the graded Zn doses applied into the soil resulted in increased TP content in seeds of faba bean cv. Zobor harvested in the stage of milk ripeness (Fig. 2). The strong statistical relationship between soil Zn content and TP amount in seeds of this faba bean cv. Zobor was confirmed (R=0.688).

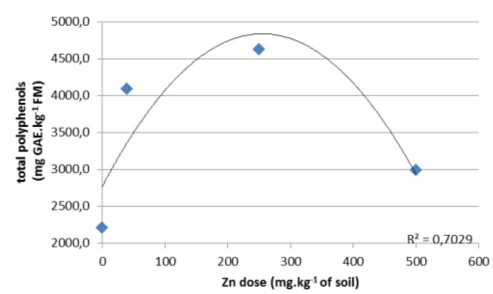

Fig. 1 Relationship between Zn input into soil and TP content in seeds of Saturn faba bean

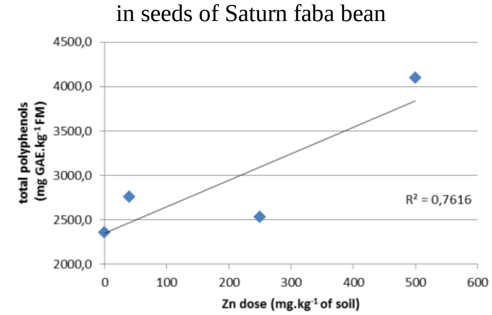

Fig. 2 Relationship between Zn input into soil and TP content in seeds of Zobor faba bean

Graded Zn doses applied into the soil resulted in increased TAC values in seeds of both of faba bean cultivars harvested in the stage of milk ripeness (Fig. 3 and 4). The strong statistical relationship between soil Zn content and TAC values in seeds of faba bean was confirmed (R= 0.913 and R= 0.908).

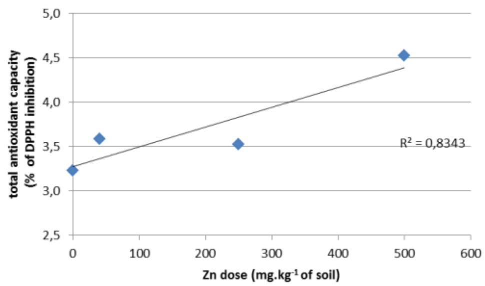

Fig. 3 Relationship between Zn input into soil and TAC content in seeds of Saturn faba bean

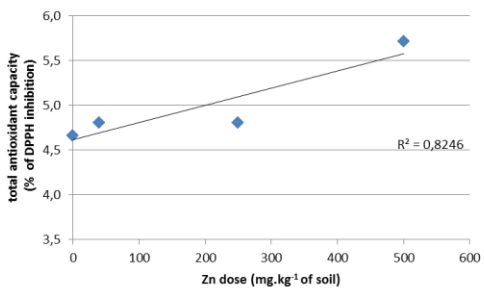

Fig. 4 Relationship between Zn input into soil and TAC content in seeds of Zobor faba bean

Fig. 5 and 6 show correlations between total polyphenol content and total antioxidant capacity values of both faba bean cultivars.

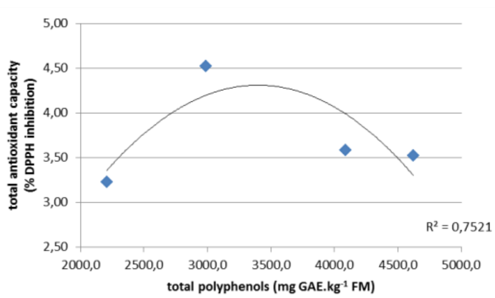

Fig. 5 Correlation between TP and TAC (faba bean Saturn)

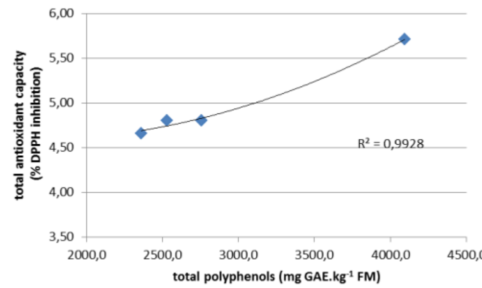

Fig. 6 Correlation between TP and TAC (faba bean Zobor)

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