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THE CONTENT OF POLYPHENOLS AND CHOSEN HEAVY METALS IN FABA BEAN (*FABA VULGARIS* MOENCH) RELATING TO DIFFERENT DOSES OF ZINC APPLICATION

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ABSTRACT
We investigated the impact of zinc on chosen heavy metals contents (Cu, Zn, Co, Cd, Pb, Cr, Ni) and polyphenols content and antioxidant capacity of chosen legume. Used soil was from Cakajovce locality (located in Nitra upland) with the neutral soil reaction suitable for the legume cultivation. Determined Cd content as well as Pb content in the soil was on the level of critical value given for the relationship between soil and plant. The values of both elements were far below threshold values proposed by European Commission. In experiment we used two cultivars of fababean – Saturn, Zobor. Seeds of fababean were harvested in milk ripeness. The different high doses of zinc (as $ZnSO_4.7H_2O$) were applied into the uncontaminated soil in model conditions. The strong statistical relationship between added Zn content in the soil and in seeds of both faba bean cultivars as well as was the total polyphenols content and the total antioxidant capacity values was confirmed. In all variants the determined Zn amount was below (Saturn) or slightly above (Zobor) the hygienic limit given by the legislative. On the other hand faba bean of both cultivars accumulated high amounts of Pb and Cd – in all variants higher than hygienic limits. The contents of all other heavy metals (Ni, Co, Cr, Cu) were lower than hygienic limits. The polyphenols content ranged from 2208 to 4622 milligrams per kilogram of fresh sample calculated as gallic acid equivalents. Faba bean shows a very small antioxidant capacity – 4.66 – 5.71% of DPPH inhibition.

Keywords: antioxidant capacity; heavy metals; faba bean; polyphenol; 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) **(Cimboláková and Nováková, 2009)**. Plants which exhibit hyperaccumulation can be used to remove heavy metals from soils by concentrating them in their biomatter. Reference Reference **Rutkowska et al. (2014)** reported the higher Zn concentration can be projected in the soil solution of acidic soils when compared with soils with neutral soil reaction.

Legumes are rich and inexpensive source of nutrietns to millions of peoples but also an excellent Zn accumulator **(Gençcelep et al., 2009)**. 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. The quantitative determination of phenolic compound content as well as their antioxidant capacity may provide valuable information in considering health-promoting properties of legume seeds **(Dalaram, 2017)**. **Turco et al., (2016)** reported that immature faba bean fractions have significantly higher phytochemical contents and display a better antioxidant activity than those of mature ones.

Therephore we investigated the effect of accumulation of heavy metals in faba bean (milk ripenness) 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 polyphenols and antioxidant activity assessed in faba bean grown in soil with increasing rates of the zinc doses.

MATERIAL AND METHODOLOGY

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 – 9 ºC. Before the realization of pot experiments the soil from this locality was sampled by valid method with pedological probe GeoSampler fy. Fisher.

Two 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 drying 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 **Lachman et al. (2003)**. Sample extracted (0.05 g to 1 mL 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 2 h 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 of 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 **Brand-Williams et al. (2005)**. 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 (At_0) with a Shimadzu spectrophotometer (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 (At_{10}). An increasing amount of antioxidants present in the methanol extract of the sample reduced DPPH inhibition according to the following equation:

$$\text{Inhibition (\%)} = [(At_0-At_{10})/At_0] \times 100$$

Statistics

All statistical analyses were carried out using the statistical software Statistica 12.0 (Statsoft, USA). Each analysis was done in six repetitions. Descriptive data analysis included mean, standard deviation. Mean comparisons between heavy metals, polyphenols content and total antioxidant activity were done by the LSD-test, $p < 0.05$.

RESULTS AND DISCUSSION

Two experiments were realised as the pot trials in the vegetation cage 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 1). 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 2).

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). The results show the mean value plus/minus the standard deviation.

In fresh seeds of cv. Saturn (Table 3) 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. The results in Table 3 show the mean value plus the standard deviation (in parenthesis).

In fresh seeds of faba bean cv. Zobor (Table 4) 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. The results in Table 5 show the mean value plus the standard deviation (in parenthesis).

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 **Hicsonmez**

Table 1 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

Table 2 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	8.44	8.74	5.5	0.29	9.06	1.8	7.3
Reference value**	**20**	**40**	**-**	**0.1**	**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**

Note: *Law No. 220/2004 valid in Slovakia.
**Decision No. 531/1994 – 540 valid in Slovakia.
***European Commission (2006).

Table 3 Heavy metals contents in the faba bean cv. Saturn (mg.kg^{-1} ±S.D.).

Variant	Zn	Cu	Ni	Cr	Pb	Cd
A (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
B (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
C (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
D (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**

Note: Limit is limit value for legumes according to the Food Codex of the Slovakia. Maximal level according to Commission Regulation 1881/2006.

Table 4 Heavy metals contents in the faba bean cv. Zobor (mg.kg^{-1} ±S.D.).

Variant	Zn	Cu	Ni	Cr	Pb	Cd
A (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
B (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
C (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
D (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**

Note: Limit is limit value for legumes according to the Food Codex of the Slovakia. Maximal level according to Commission Regulation 1881/2006.

Table 5 Total polyphenol content (mg GAE.kg^{-1} ±S.D.) and total antioxidant capacity (% of DPPH inhibition±S.D.).

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

et al. (2012) 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 **Haciseferoğullari et al. (2003)** 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. **Dalaram et al. (2016)** showed the content of the metals studied in similar uncontamined (from point of view of heavy metals content), with the exception of cadmium, not exceed the maximum permissible value in legumes.

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 **Gadd (1992)** and **Giller et al. (1998)** 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 **(El-Sokkary and Sharaf, 1996)**. 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 **(Rattan et al., 2005)**.

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 5. 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 **Chaieb et al. (2011)** 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. **Turco et al. (2016)** reported results of polyphenols determination in faba bean from different countries – Algeria (4490 – 42440 mg.kg^{-1}), Tunisia

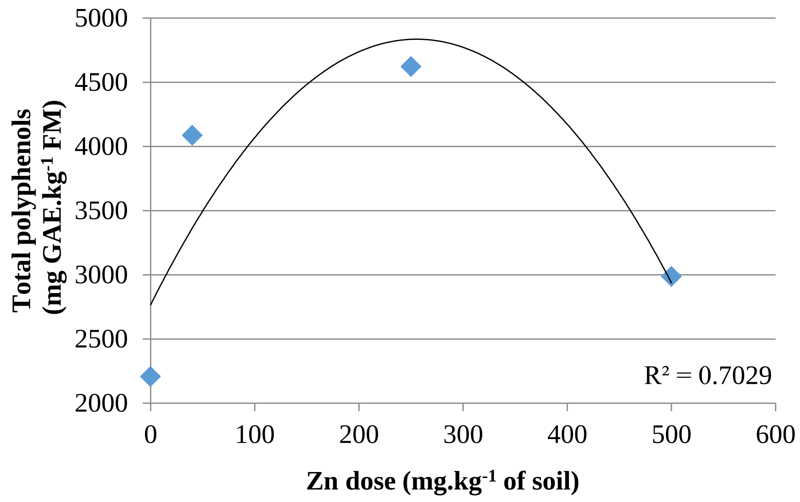

Figure 1 Relationship between Zn input into soil and TP content in seeds of faba bean cv. Saturn.

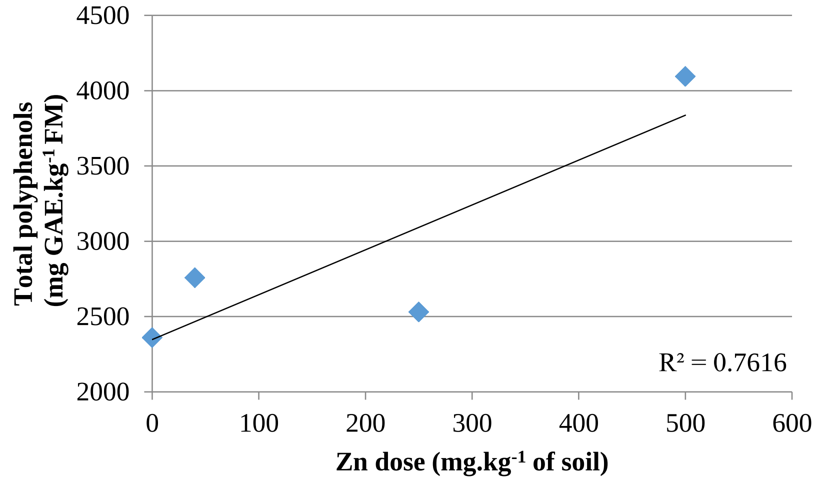

Figure 2 Relationship between Zn input into soil and TP content in seeds of faba bean cv. Zobor.

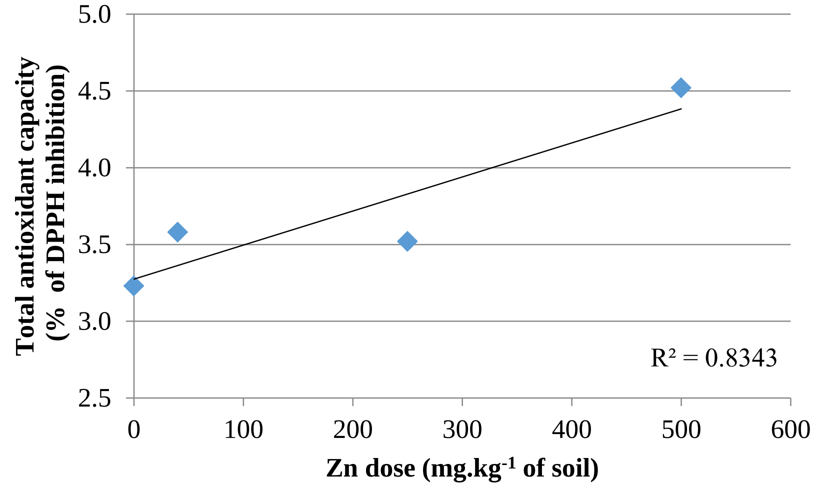

Figure 3 Relationship between Zn input into soil and TAC content in seeds of faba bean cv. Saturn.

(16980 – 67470 mg.kg^{-1}), Canada (5590 – 37760 mg.kg^{-1}), Chile (820 – 1340 mg.kg^{-1}). In addition to genetic factors, total phenolic content in seeds is modulated by other factors such as growing stage (vegetative, reproductive and mature).

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 (Figure 1),

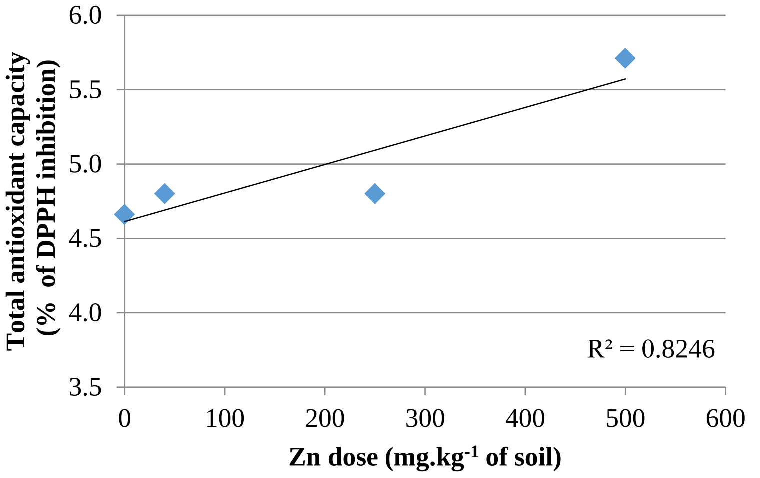

Figure 4 Relationship between Zn input into soil and TAC content in seeds of Zobor faba bean.

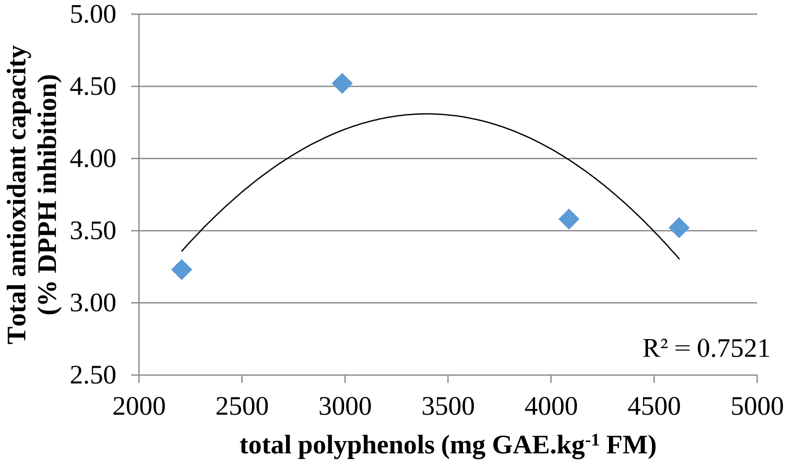

Figure 5 Correlation between TP and TAC (faba bean cv. Saturn).

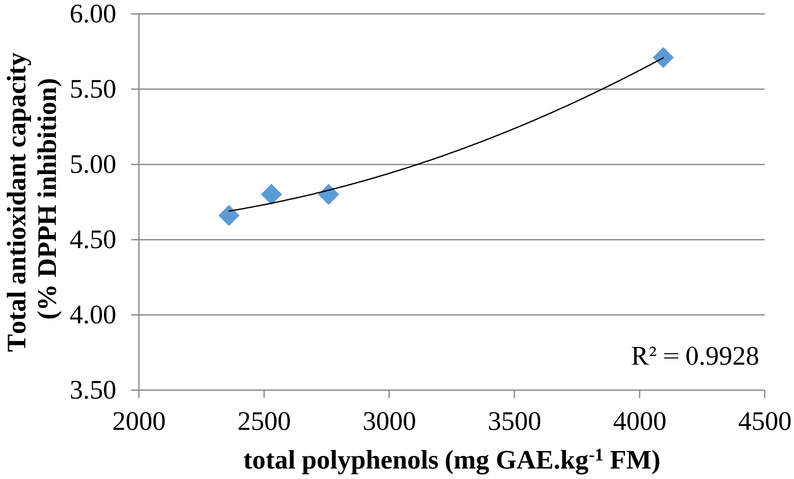

Figure 6 Correlation between TP and TAC (faba bean cv. Zobor).

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 (Figure 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).

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 (Figure 3 and Figure 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).

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

CONCLUSION

Legumes are considered to be a promising crop in the view of human nutrition. Therefore it is important to obtain the complex knowledge about their safety from the aspect of heavy metal content as well as about the bioactive important components and their possible entry into the food chain. 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. With increased Zn doses applied into the soil in the model conditions the TP contents and total antioxidant capacity values determined in seeds of both of faba bean cultivars Saturn and Zobor harvested in the stage of milk ripeness were increased. The strong statistical relationship between soil Zn content and TP amount and TAC in seeds of faba bean cultivars was confirmed.

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