ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 7-13

Changes of Some Biochemical and Physiological Parameters in Capsicum annuum L. as a Consequence of Increased Concentrations of Copper and Zinc

Snezana T. Stavreva-Veselinovska, Jordan B. Zivanovik, Milena M. Djokic

University "Goce Delcev", Stip, MACEDONIA
E-mail: snezana.stavreva@ugd.edu.mk, jordan.zivanovik@ugd.edu.mk
milena.gokik@ugd.edu.mk

Abstract. Soil cultures of peppers were (*Capsicum annuum* L.*)* cultivated. The first leaves of young plants in the stage of forming were treated with excessive concentrations of $ZnSO_4 \cdot 7H_2O$ in four different concentrations (mg/kg): 1.0; 5.0; 10.0 and 20.0 while the control group of plants was treated with water only. Parallel to this, a part of the plants were treated with excessive concentrations of $CuSO_4 \cdot 5H_2O$ again in four different concentrations: 0.5; 1.0; 5.0 and 10.0, while the control group of plants was treated with water only. The material for the analysis was taken at the end of the vegetation period, in the phase of bearing fruit, and then it was dried to absolute dry mass at the temperature of 60-80^{o}C. The dry plant material is broken up into small pieces and used for analyzing the phenol compounds. Raw material is used for determining the contents of Chlorophyll and vitamin C.

Key words: heavy metals, copper, zinc, *Capsicum annuum* L , phenol compounds, chlorophyll

Introduction

Soil is fundamental and irreplaceable part of the environment and its pollution cannot be avoided. Even though the soil has great buffer capacity in relation to outer influences, the functioning of this capacity can be disturbed, and this in turn represents a considerable problem of today's modern society. Plants are an important indicator of soil pollution with heavy metals.

Heavy metals and their influence on mineral feeding of plants

The chemical substances that can be commonly found in nature every day (air, water, soil) are increasing in number. By origin, they can be natural products, but not rarely also synthetic, or they are the products of chemical transformation of natural products. Isolated as pure chemical substances they possess certain characteristics that make them important and are the basis of their everyday application. These substances are usually found in soil but in various quantities. One type of these matters is elements that are found in soil in such small quantities that they are called elements in traces or microelements. Other elements, found in soil in traces but not necessary to plants, in great quantities can be harmful and dangerous for plants, man and animals. This group consists of toxic elements, and the above-

mentioned groups are made up mostly of such heavy metals. These two terms comprise a group of metals that can pollute the soil and the environment. Unlike water and air pollution, soil pollution with heavy metals is not easy to determine and is different for different soil types.

Research goal

Interest in growing fruits of pepper, is on the rise, due to its high content of bioactive substances and antioxidants. The research showed the impact of heavy metals and different variation of several components with antioxidant properties. Through the concentration of synthesized substances with antioxidant capabilities can be observed the influence of toxic metals.

Starting from the previous, and having in mind the toxic influence of the excessive concentrations of heavy metals upon some morpho-physiological and anatomic parameters in a number of plant species, the goal of this research was do examine the effects of different concentrations of copper and zinc on the contents of photosynthetic pigments, vitamin C and bioflavonoid (anthocian and phenol).

Material and methods

The pepper culture (*Capsicum annuum* L.*)* cultivated in experimental conditions was used in the research. During the growing phase, the young leaves were treated with excessive concentrations of $ZnSO_4 \cdot 7H_20$ with 4 different concentrations (mg/kg): 1.0; 5.0; 10.0 and 20.0 while the control group of plants was treated only with water. Simultaneously a part of the plants were treated with excessive concentrations of $CuSO_4 \cdot 5H_2O$, again with 4 different concentrations: 0.5; 1.0; 5.0 and 10.0 while control plants were treated only with water. The material for analysis was taken toward the end of vegetation, in the phase of bearing fruit, and it was dried afterwards until it was dry mass at the temperature of 60-80°C. The dry plant material is chopped up and used for analysing the phenol compounds. The fresh material is used for determining the content of chlorophyll pigments and vitamin C.

Determination of the content of chloroplast pigments using spectrophotometric method

200 mg fresh leaf mass of pepper (*Capsicum annuum* L.) were measured on an analytic scale. They were then transferred into a porcelain mortar where it is macerated in the presence of 85% solution of acetone. The procedure is repeated until the leaf mass is completely decolorized. The resulting extracts are kept in the dark in order to prevent the destruction of the chlorophyll molecules. The extracts are photo-metrically measured at the wave length of:

Chlorophyll a (*Chl* a) - 665 nm
Chlorophyll b (*Chl* b) - 650 nm
Carotenods – 452.5 nm

A pure acetone solution is used as a blind sample.

The concentration (content) of chlorophylls in the solution is calculated with the following formulas *MECKINNEY* expressed in mg/l:

Chl a = 16.5 ∗ A 665 – 8.3 ∗ A650
Chl b = 33.8 ∗ A650 – 12.5 ∗ A665
Chl (a + b) = 4.0 ∗ A665 + 25.5 ∗ A650

Determination of Vitamin C content according to 2, 6-dichlorophenolindophenol method

First the available leaf mass for this analysis is measured on an analytical scale. The plant material is macerated with 5-6 drops of 3% threechlor acid solution; it is quantitatively filtrated in a 100 ml lab dish and is filled to the mark with 3% measured by means of a mensure and this is moved into solution of CCl_3COOH. Out of this solution 20 ml are an Erlenmeyer dish where it is titrated with 2,6-dichlorfenolindofenol until slight pink coloration occurs which lasts as long as one minute. The concentration of the titrants is 0,001 mol/dm^3. The solution mass of 2,6-dichlorfenolindogenol is calculated with the

following formula:

$$m = V \cdot C \cdot M$$

The mass got from the indicator is dissolved in a measuring 100 ml lab dish.

To calculate the percentage (%) of vitamin C in the plant material the following formula is used:

$$\%C = \frac{V \bullet 0{,}001 \bullet 176}{m}$$

V- volume of used ml of 2,6-dihlorfenolindofenol;

0,001 - concentration of 2,6-dihlorfenolindofenol;

176 - molar mass of ascorbic acid;

M - grams of the taken material for analysis.

Determination of the anthocianines content

The dried fruit mass used for anthocianine extraction is first measured and we take 1-10 g (depending on the anthocianine content). The quantity taken is transferred into a 100 ml dish and filled to the mark with 1% of HCL solution in methanol. After the extraction is finished (30 minutes in the dark), it is filtered through filter paper. From the resulting filtrate we take 5 ml and put it into the measuring dish of 50 ml and fill it to the mark with a buffer pH-1.

This solution is used for the analyses are measured in relation to the control of the spectrophotometer at the wavelength of 510 nm.

The following formula is used for calculation:

$$A = \frac{E - PH_1 \bullet V_1 \bullet V_2}{m_1 \bullet m_2 \bullet V_1}$$

V_1 - volume of the filtrate;

V_2 - volume of the extract;

m_1- fresh mass of the plant material;

m_2 - dry mass of the plant material.

Determination of phenol and flavonoid content according Folin−Ciocalteu method

The extraction of phenols and flavonoids begins with macerating of the fruit mass with 3ml 80% methanol and is incubated 30 minutes at 4°C in an ultrasonic bath. After that the extract is centrifuged for 10 minutes at 13700 rpm. After centrifuging 2 ml of the supernatant is collected into specially labelled test tube, and methanol is again added to the residue (grounds) and re-extraction and centrifuging is performed; then another 2 ml are collected. The procedure is performed after 1ml Folin−Chioclateau reagent and 800 µl 0.7 MNa_2CO_3 are added to 1 ml extract. The mixture incubates for 5 minutes at room temperature.

The absorption of the total phenols is measured at 765 nm and for flavonoids at 425 nm.

The solution of catehin (0.4 mg/ml) is used to prepare the standard curve.

The formula for calculating is as follows:

$$\text{A}\mu\text{g catehin} = \frac{\sum A_{st}}{\sum AC_{st}}$$

Aµg-catehin for total phenols (765 nm)=0.332
Aµg-catehin for flavonoids (425 nm)=0.161

Preparation of the original solution: 50 ml of dry plant material is dissolved in a 25 ml lab dish with several drops of 80% methanol (also possible 100%), and is filled to 25 ml with methanol -FV1. 0.5 ml is taken for determination. If concentration is high, it is diluted. The calculation is done according to the following formulas:

$$Cmg/L = \frac{Aproba}{A(1\mu g_{katehin}) \bullet FV} \bullet DF / 1000(\mu g / mg)$$

$$DF = \frac{FV}{V}.$$

Results and Discussion

The following results were got after the analyses made in pepper (*Capsicum annuum L.*) concerning certain biochemical-physiological parameters (Table 1).

Table 1. Content of photosynthetic pigments (mg/100g wet weight) in pepper plant material (*Capsicum annuum* L), treated with $ZnSO_4 \cdot 7H_2O$.

Treatment with $ZnSO_4 \cdot 7H_20$(mg/kg)	Chl a	Chl b	Chl (a+b)
Control	204.5	340.2	544.7
1.0	181.0	278.2	459.2
5.0	193.0	318.5	510.9
10.0	184.5	299.5	481.7
20.0	197.2	310.0	507.0

Zinc is a significant plant nutrient in limited quantities. If it is added in concentrations greater than optimum, it gets toxic. The presence of greater quantities of reductive metals such as Cu, Fe or Zn in plants causes oxidative damage (LUNA *et al.,* 1994) as well as lipid per-oxidation and anti-oxidative protection (GORA & CLIJSTERS, 1989). A negative effect can be noticed in our results caused in plants by Zn through the reduction of the chlorophyll a and b biosynthesis, when it is applied in concentrations exceeding the optimum.

Zn mainly acts as an inhibitor of the photosynthetic electronic transport (KAPPUS, 1985) causing reduction of the maximum efficacy capacity of PS2 (F_v/F_m), and in quantum gain of electric transport through PS2.

The same parameters were examined in *Lolium perenne* and it was noticed that bigger concentrations of Zn were first seen in its growth inhibition. The increased concentration results in concentration reduction of Ca, K, Mg and Cu, reduction in quantum gain from the electric transport through PS2, as well as the efficacy and photosynthetic energy conversion compared to the control plants (MAKSYMIEC & BASZYNSKI, 1996). However, it was noticed that in this plant only the biggest concentration of 50 mg/kg results in such an effect, which in turn shows that these plants have great power of protection from high concentration of heavy metals. Nevertheless, the number of plants that accumulate Zn well is small.

Table 2 present the results of the analysis of pepper (*Capsicum annuum L.*) that was previously treated with $CuSO_4 \cdot 5H_2O$.

Table 2. Content of photosynthetic pigments (mg/100g) in pepper plant material (*Capsicum annuum* L.)

Treatment with $CuSO_4 \cdot 5H_20$(mg/kg)	Chl a	Chl b	Chl (a+b)
Control	204.5	340.2	544.7
0.5	212.3	332.7	545.0
1.0	174.5	270.7	444.2
5.0	210.5	361.7	572.0
10.0	219.5	419.7	639.0

From the results in Table 2 of the analyses performed on pepper (*Capsicum annuum* L.) we can notice that in higher concentrations at the beginning of the experiment copper shows a stress effect on the treated plants which in turn leads to the reduction in chlorophyll a and b bio-synthesis; later, during the application of greater concentrations a kind of plant

adaptation occurs, probably because of the building in of the Cu into the compounds – participants in photosynthesis, mostly plastocyanine.

Here higher concentration of Cu leads to greater chlorophyll a and b synthesis. We can notice that, compared to zinc, pepper much better adapts to copper.

Copper is an important for plants and especially for photosynthesis as a process because of its role in the transport of electrons as a constitutive part of cyto-chrome.

It mostly leads to the inactivation of Rubesko and phosphoenol piruvat-carboksilase (PEPC), through an interaction with SH-groups (LIDON & HANRIQUES, 1991). It stimulates lipid peroxidation (SANDMANN & BÕGER, 1980), which continues with serous damage of tilacoid membranes. It mostly affects the reduction of the chlorophyll influencing its synthesis as well as its degradation (VANGROUSVELD & CLIJSTERS, 1994).

A great number of authors worked on the same analyses in different plants. *Hordeum vulgare* is one of the analysed plants where the toxic effect of copper was confirmed. Here it was noticed that Cu causes a strong lipid peroxidation (SANDMANN & BÕGER, 1980), which again results in the destruction of the tilacoid membranes and the reduced synthesis of chlorophyll a and b; the reduction is connected with the existing limitations of the tilacoid membranes (VASSILEV *et al.,* 2002). It was also noticed that the synthesis of ethylene is strengthened (LIDON & HANRIQUES, 1991), also noticed in spinach, rice, etc.

As for the content of ascorbic acid in the course of treating pepper with $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$, the following can be stated:

Table 3. Content of vitamin C in fresh pepper plant material (*Capsicum annuum L*)

Treated plants	Control	$ZnSO_4 \cdot 7H_20$ 20mg/kg	$CuSO_4 \cdot 5H_2O$ 10 mg/kg
(%)vitamin C	0.0911	0.206	0.1139
ml/100g	26.200	86.17	30.730

Looking at the results in Table 3 got from the pepper we can see that an increased synthesis of vitamin C occurs as well as of the enzymes taking part in protecting the plant from oxidation that is caused by high concentrations of copper and zinc. Ascorbic acid is the main anti-oxidant in the photosynthetic and non-photosynthetic tissues where it directly reacts with ascorbat oxidat.

Similar to this enzyme is ascorbat-peroxidase that catalyzes the de-toxication of H_2O_2 (NOCTOR & FOYER, 1998). We have already said that when a higher concentration of Zn or Cu is applied in plants it mainly causes lipid peroxidation of the membrane leading to freeing a great number of free radicals. The synthesis of ascorbic acid is used by plants for protection from the created free radicals.

The analysis made in *Lolium perenne* showed an increase in the activity of SOD-super-oxide desmutase which takes part in the degradation of the formed super-oxide radical as well as of ascorbat oxidase, monohydroascorbat reductase, dehydro-ascorbat reductase and glutation reductase. SOD increases up to 100% when $ZnSO_4$ reaches the concentration of 50 mM (OUARTLI *et al.,* 1997). It can be seen in our results that the highest concentration of Zn also mostly increases the concentration of vitamin C.

The results of the research analysis made on pepper concerning the concentration of anthocianines lead to the following:

Table 4. Content of anthocians (mg/l00g) in the fruit of red pepper treated with different concentrations of $ZnSO_4 \cdot 7H_2O$

	Control	1,0 mg/kg	5,0 mg/kg	10 mg/kg	20 mg/kg
Anthocians	42	96.98	84.095	45.91	47.938

Table 5. Content of anthocians (mg/l00g) in fruit treated with different concentrations of $CuSO_4 \cdot 5H_2O$

	Control	0,5 mg/kg	1,0 mg/kg	5,0 mg/kg	10 mg/kg
Anthocians	42	255.485	1032	1302	71.265

From the analyses made and the result in Table 4 it can be concluded that during treatment of plants with Zn there is a reversely proportionate dependence between the applied concentration of respective heavy metals and the anthocians content being synthesized; the least concentration leads to greatest synthesis; still, all the values are bigger than in control plants.

Copper that also enters the anthocianine synthesis increases the anthocianine synthesis much more compared to the control group of plants according to Table 5; however, in the last case we notice a great fall in the anthocianine content and we can come to the conclusion that the plant somehow stops fighting against the negative influence of heavy metals. When looking at the comparative results of the influence of copper and zinc in both Table 4 and 5 on the anthocianine synthesis it can be said that Zn does not cause significant stress effect in pepper.

Table 6. Content of phenols (mg/g) and flavonoids (mol/g)in plants treated with $ZnSO_4 \cdot 7H_2O$

	Phenols mg/g	Flavonoids mol/g
Control	21.540	7.910
20mg/kg $ZnSO_4 \cdot 7H_2O$	17.006	8.470

Anthocianines represent compounds that, among other, also show anti-oxidative characteristics, protecting the plants from the formed free radicals (Lee and Gould, 2002).

Anthocianine, compared to other components, represents the best indicator of the oxidative stress resulting in plants under the influence of heavy metals. As a mechanism protecting from the toxic influence of high concentrations of Zn and Cu it is synthesized in plants in great concentrations if compared to the control plant.

Table 7. Content of phenols (mg/g) and flavonoids (mol/g) in plants treated with $CuSO_4 \cdot 5H_2O$

	Phenols mg/g	Flavonoids mol/g
Control	21.540	7.910
10mg/kg $CuSO_4 \cdot 5H_2O$	18.270	8.810

Based upon these results shown in Table 6 and 7 we can state that, generally, both zinc and copper cause a fall in the total content of phenols (Table 6 and 7) compared with the controle, while the content of flavonoids (Table 6 and 7) and anthocianines (Table 4 and 5) is increased. There is a directly proportionate dependence between the concentration of heavy metals and the contents of flavonoids and anthocianines, and a reversely proportionate dependence with the total phenols.

Phenolsand flavonoids are important anti-oxidants. Szent-Györgyi, Nobel Prize winner who isolated the ascorbat demonstrated that flavonoids behave in the same manner as the ascorbat. Their synthesis can be induced by biotic and abiotic factors (DIXON & PAIVA, 1995). Phenols are considered tobe anti-oxidants the function of which is to help the primary ascorbat-dependent anti-oxidative system in plants (YAMASAKI *et al.*, 1999).

Conclusions

The results shown clearly indicate the negative effect that heavy metals have on plant material of *Capsicum annuum*. This leads to the following conclusions:

1. All the applied concentrations of heavy metals result in toxic symptoms in plants, and

the seriousness of the damage depends on the plant type, kind of the pollutant, manner of application, concentration, etc.

2. After treating with Zn and Cu a reduction of the photosynthetic activity was noticed, i.e. the chlorophyll pigments.

3. A rapid increase of the vitamin C synthesis was noted as well as of anthocianines, phenols and flavonoids because of an anti-oxidative defence of the plant from free radicals.

References

DIXON R., N. PAIVA. 1995. Stress-induced phenylpropanoid metabolism. - *Plant cell*, 7: 1085-1097.

GORA L., H. CLIJSTERS**.** 1989. Biochemical and physiological aspects of ethylene production in lower and higher plants. - In: Clijsters H, De Proft M, Marcelle R, Van Poucke M, (Eds.) Dordrecht, Kluwer Academic Publishers, pp. 219–228.

KAPPUS H. 1985. Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. - In: Sies H. (Ed.) *Oxidative stress.* London: Academic Press, 273–310.

LEE, D., K. GOULD. 2002. Anthocyanins in leaves and other vegetative organs: an introduction. – In: Gould K., D. Lee. (Eds.) *Anthocyanin in Leaves. Advances in Botanical Research,* Academic Press, London, pp 1–16.

LIDON F., S. HENRIQUES. 1991. Limiting step on photosynthesis of rice plants treated with varying copper levels. - *J. Plant Physiology,* 138: 115-118.

LUNA C., C. GONZALEZ, V. TRIPPI. 1994. Oxidative damage caused by an excess of copper in oat leaves. - *Plant Cell Physiology*, 35**:** 11–15.

MAKSYMIEC W, T. BASZYNSKI**.** 1996. Chlorophyll fluorescence in primary leaves of excess Cu-treated runner bean plants depends on their growth stages and the duration of Cu-action**.** -*Journal of Plant Physiology*, 149**:** 196–200.

NOCTOR G., C. FOYER. 1998. Ascorbate and glutathione: Keeping active oxygen under control. - *Ann. Rev. Plant Physiol. Plant Mol. Biol*., 49: 249-279.

OUARITLI O., H. GOUIA, M. GHORBAL**.** 1997. Responses of bean and tomato plants to cadmium: growth, mineral nutrition and nitrate reduction. - *Plant Physiology and Biochemistry*, 35**:** 347–354.

SANDMANN G., G. BÕGER. 1980. Copper-mediated lipid peroxidation processes in photosynthetic membranes. - *Plant Physiology,* 66: 797-800.

VANGROUSVELD J., H. CLIJSTERS. 1994. Toxic effects of metals. - In: Farago M. (Ed.), *Plants and the chemical elements. Biochemistry, uptake, tolerance and toxicity,* VCH Publishers, Weinheim, Germany, pp. 150-177.

VASSILEV A., F. LIDON, M. DOCÈUMATOS, J. RAMALHO, I. YORDANOV. 2002. Photosynthetic performance and some nutrients content in cadmium and copper-treated barley plants. - *J. Plants Nutr.,* 25(11): 2343-2360.

YAMASAKI H., S. TAKAHASHI, R. HESHIKI. 1999. The tropical fig *Ficus microcarpa* L.f.cv. Golden Leaves lacks heat-stable dehidroascorbate reductase activity. - *Plant Cell Physiology,* 40: 640-646.

Received: 01.05.2010
Accepted: 19.07.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House