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ANTIOXIDANT ACTIVITY OF TOBACCO (*Nicotiana tabacum* L.) IN INTOXICATION WITH HEAVY METALS

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ABSTRACT

For determination of bio-physiological parameters, fresh leaf material of two tobacco varieties (Prilep P-156/1 and Yaka Yv-125/3) was used. Soil cultures were grown in controlled experimental conditions, in a glasshouse of the Institute of Biology (Faculty of Natural Sciences and Mathematics-Skopje). For each treatment ten plants were taken and were treated with four concentrations of heavy metals: $CuSO_4$ x $5H_2O$ (0.25 mg/kg soil, 0.5 mg/kg soil, 1mg/kg soil, 5 mg/kg soil), $CdSO_4$ x $8H_20$ (0.1 mg/kg soil, 0.2 mg/kg soil, 0.4 mg/kg soil and 0.8 mg/kg soil), $Pb(NO_3)_2$ (50 mg/kg soil, 100 mg/kg soil, 200 mg/kg soil, 400 mg/kg soil) and the excess concentration (unallowable for global use) of *Antracol WP-70* (0.2 mg/kg soil, 0.4 mg/kg soil, 0.8 mg/kg soil and 1.6 mg/kg soil). *Antracol WP-70* is fungicide of the dithiocarbamate group, with zinc as its basic constituent. In the same time, a trial with control group of ten tobacco plants was set up. Of ten fully matured leaves from lower, middle and upper leaf belts was collected for analyses. All analysis was performed in triplicate. For the aim of this paper, leaves from the upper belt (III insertion) were used. The accent was put on the analyses of chloroplast pigments content (spectrophotometric method of Holm & Wettstein, 1958) catalase activity (titrimetric method of Bach & Oparin) and anthocyanins content (spectrophotometry at 510 nm).

Key words: intoxication, tobacco, heavy metals, fungicide, chloroplast pigments

АНТИОКСИДАТИВНА АКТИВНОСТ КАЈ ТУТУНОТ (*Nicotiana tabacum* L.) ПРИ ИНТОКСИКАЦИЈА СО ТЕШКИ МЕТАЛИ

Контаминацијата со тешки метали претставува приоритетен проблем во современото општество кој иницира низа малформации, посебно кај растенијата, во услови на силна интоксикација со истите. Целта на оваа студија е одредување на биохемиско-физиолошките параметри на тутунот (*Nicotiana tabacum L*) од реколтата 2008 кај две ориенталски сорти (*Прилеп П-156/1* и *Јака Јв-125/3)*. Растенијата се одгледувани во контролирани, експериментални услови во стакленик на Ботаничката градина при Институтот за биологија (Природно-математички факултет, Скопје). За секој третман земени се по десет тутунските растенија кои беа третирани со тешки метали (бакар, кадмиум и олово) и фунгицид (*Antracol WP-70*) во четири различни концентрации. Истовремено е поставена контролна група од десет тутунски растенија. За анализа се земани по десет целосно зрели листови од појаси на долен, среден и горен лист.

Сите испитувања се направени во три повторувања. За овој труд анализиран е материјал од горниот лист, или трета инсерција од вегетацискиот период на растенијата. Во постапките за анализа акцентот беше ставен на испитувањето на содржината на хлоропласни пигменти (спектрофотометриски метод по Holm и Wettstein, 1958), активноста на каталазата (титриметриски метод по Бах и Опарин), како и содржината на антоцијани (спектрофотометрирање на 510nm). Ефектите од интоксикацијата се одредени преку споредба на третираните садови со контролната група на тутунски растенија, со цел да се потврдат сознанијата за влијанието на тешките метали врз карактеристиките на тутунот како комерцијалн.

Клучни зборови: интоксикација, тутун, тешки метали, фунгициди, хлоропласни пигменти

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) was cultivated thousands of years ago. At first it served as a medicinal plant and later it started to be used as commercial raw and agricultural crop. In the last few decades its application has been broadened and it became widely used as a model-system in plant cells cultures and in genetic engineering researches. Due to its huge economic importance, it became an object of many investigations both of its origin and evolution and of its genetic and structural organization.

Three main types of tobacco grown in this area are Prilep, Yaka and Basmak.

Heavy metals present a strong stress factor to plant metabolism. Their content in plants depends on genetic specificity of the species, their selective abilities for metals uptake and the level of environment pollution. According to William (1981), heavy metals can affect the activity of some enzymes. They function as antimetabolites, create resistant precipitations or chelates in constituent parts of the cells, act as catalysts in decomposition of plant metabolites, change the permeability of cell membrane or replace the major structural chemical elements in the cell. Cadmium, nickel and thallium show high mobility and can be easily removed from the plant, while other elements (mercury, vanadium, lead and zinc) have an ability to accumulate in plant organs.

Cadmium provokes oxidation, but unlike other heavy metals, e.g. Cu, it does not directly influence the production of reactive types of oxygen. Activation and slowing of the antioxidative enzymes depends not only on intensity of the effect and its duration but also on the type of tissue and plant age. Lead can provoke inhibition of plant growth and development, prolongation of root, germination and seed development, division of cells, photosynthesis, transpiration, chlorophyll synthesis, growth of ethioplates and lamellar organization of the chloroplasts (Wozny & Jerczynska, 1991).

Copper uptake by plants is in small amounts, mostly as Cu-ions in chelate form. With increased concentration of copper in the environment, however, the plants ability for absorption of Cu also increases. Cu-ions are absorbed not only by plant roots, but also by its above-ground parts. Plant sensitivity to copper insufficiency differs, and oat, wheat, barley, tobacco and spinach belong to the group of very sensible. Fungicides, insecticides and pesticides can be potential toxicants.

Uniformity and constancy of biological-morphological and technological-industrial properties are of special importance for tobacco quality. For improvement of quality, especially of tobacco types Prilep (P-156/1) and Yaka (Yv-125/3), the effect of heavy metals (Cu, Cd, Pb) and fungicide *Antracol WP-70* was investigated through analysis of the chloroplasts and content and catalase activity. The effects of intoxication were determined through comparison of treated crops with the control group of tobacco plants.

MATERIAL AND METHODS

For determination of bio-physiological parameters, fresh leaf material of two tobacco varieties (Prilep P-156/1 and Yaka Yv-125/3) was used. Soil cultures were grown in controlled experimental conditions, in a glasshouse of the Institute of Biology (Faculty of Natural Sciences and Mathematics-Skopje). Tobacco plants were treated with four concentrations of heavy metals: $CuSO_4$ x $5H_2O$ (0.25 mg/kg soil, 0.5 mg/kg soil, 1mg/kg soil, 5 mg/kg soil), $CdSO_4$ x $8H_20$ (0.1 mg/kg soil, 0.2 mg/kg soil, 0.4 mg/kg soil and 0.8 mg/kg soil), $Pb(NO_3)_2$ (50 mg/kg soil, 100 mg/kg soil, 200 mg/kg soil, 400 mg/kg soil) and the excess concentration (unallowable for global use) of *Antracol WP-70* (0.2 mg/kg soil, 0.4 mg/kg soil, 0.8 mg/kg soil and 1.6 mg/kg soil). *Antracol WP-70* is fungicide of the dithiocarbamate group, with zinc as its basic constituent. In the same time, a trial with control group of tobacco plants was set up. Material from lower, middle and upper leaf belts was collected for analyses. For the aim of this paper, leaves from the upper belt (III insertion) were used. The accent was put on the analyses of chloroplast pigments content (spectrophotometric method of Holm & Wettstein, 1958) catalase activity (titrimetric method of Bach & Oparin) and anthocyanins content (spectrophotometry at 510 nm).

RESULTS AND DISCUSSION

In addition to heavy metals intoxication, it was determined that tobacco itself is a source of high amounts of Cu and Zn, in concentrations of 12.90 mg/g and 55.62 mg/g dry mass weight, respectively (Massadeh et al., 2003).

Results of the bio-physiological analyses are presented in tables and figures.

Table 1.Catalase activity in dry tobacco leaves of varieties Prilep P-156/1 and Yaka Yv-125/3, upper belt (III incertion)

Treatment	Catalase activity Prilep P-156/1 (mg H_2O_2)	Catalase activity Yaka Yv-125/3 (mg H_2O_2)
Control	1.35	1.09
Cu I	1.02	1.47
Cu II	0.26	0.93
Cu III	0.56	0.34
Cu IV	1.02	0.69
Pb I	0.63	1.96
Pb II	0.29	0.46
Pb III	0.24	0.66
Pb IV	0.22	0.64
Cd I	0.49	0.46
Cd II	0.9	0.43
Cd III	0.25	0.51
Cd IV	0.49	0.17
Fungicide I	1.21	0.45
Fungicide II	0.64	0.85
Fungicide III	1.06	0.21
Fungicide IV	1.96	1.08

In relation to its structure, catalase is a proteide which role is to decompose the highly toxic H_2O_2. Its translocation and negative impact on plant metabolism is difficult to follow (Willekens et al., 1997). Catalase activity is particularly important in the case of intoxication with heavy metals, when the production of hydrogen peroxide is increased.

Catalase activity reflects the ability of organism to supply strong protective mechanism against unfavorable environmental conditions. In this context the photosynthetic activity of plants should be mentioned (Zeltich, 1992). Investigations of cadmium, copper, iron and zinc effects on the activity of antioxidant enzymes in *N. tabacum* (Sokolnik et al., 2007) revealed that these elements stimulated the activity of dismutase but reduce the catalase activity. It can be noted that catalase activity is reduced when tobacco is treated with Cu, Cd and Pb, but it is also negatively affected by the application of fungicides. The catalase activity shows higher values in the following cases: the fourth concentration of fungicide for variety P-156/1 (1.96 mg dissolved H_2O_2), the first concentration of Cu for Yv-125/3 (1.47 mg dissolved H_2O_2) and the first concentration of lead (1.96 mg dissolved H_2O_2). In all other treatments of tobacco plants with metals, the level of catalase activity is lower compared to the control group.

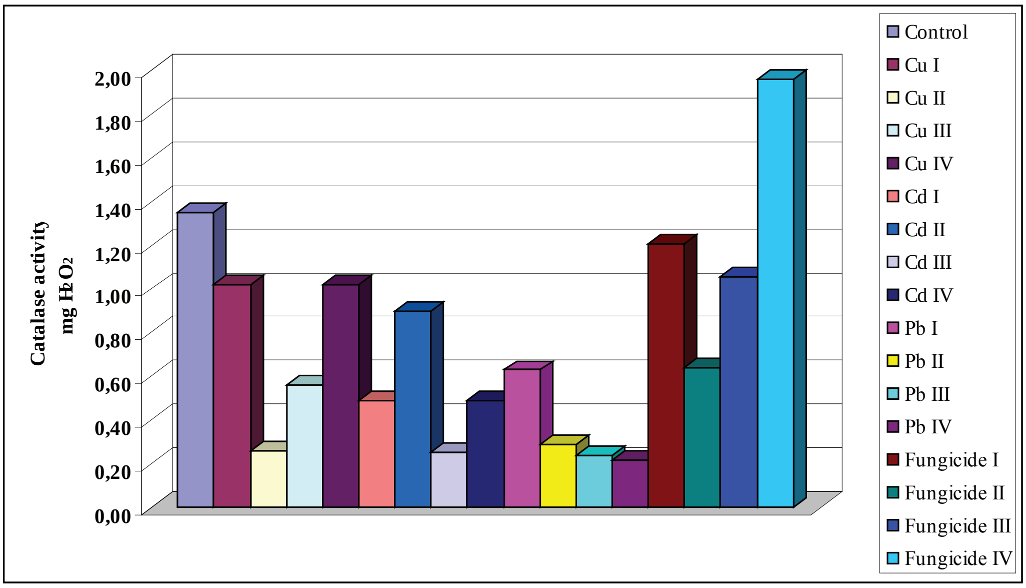

Figure 1. Catalase activity in dry tobacco leaves of variety Prilep P-156/1, upper belt (III incertion)

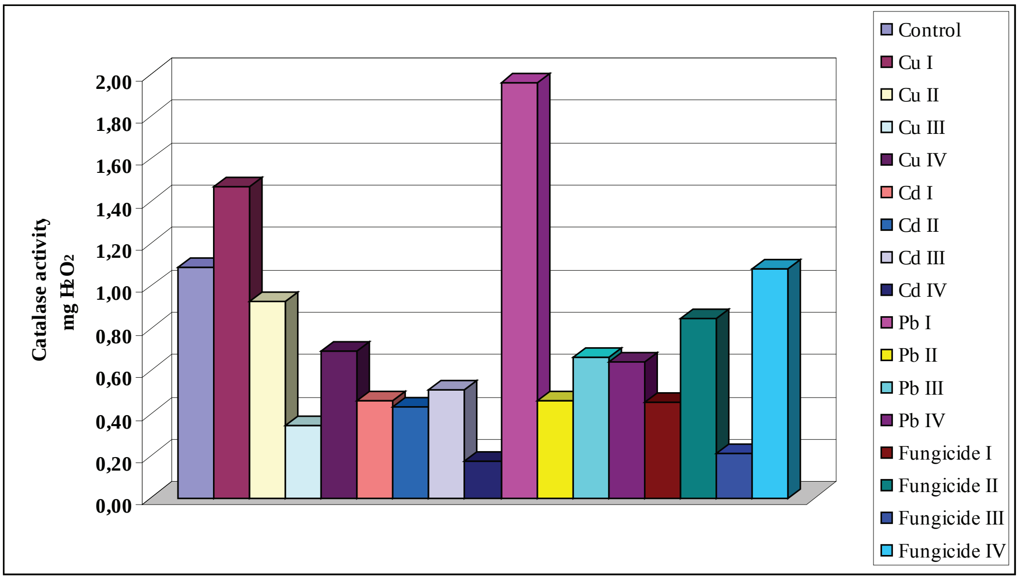

Figure 2. Catalase activity in dry tobacco leaves of variety Yaka Yv-125/3, upper belt (III incertion)

Table 2. Content of chloroplast pigments (mg/g) in fresh material of tobacco leaves of variety Prilep P-156/1, upper belt (III incertion)

Treatment	Chl-a (mg/g)	Chl-b (mg/g)	Chl-a+b (mg/g)	Carotenoids (mg/g)
Control	0.68	0.67	1.34	0.20
Cu I	0.30	0.35	0.65	0.13
Cu II	0.90	0.41	1.32	0.14
Cu III	0.19	0.30	0.50	0.10
Cu IV	0.12	0.36	0.48	0.11
Pb I	0.34	0.44	0.79	0.16
Pb II	0.54	0.56	1.10	0.19
Pb III	0.05	0.49	0.80	0.16
Pb IV	0.22	0.26	0.49	0.10
Cd I	0.26	0.27	0.53	0.14
Cd II	0.33	0.34	0.67	0.16
Cd III	0.27	0.36	0.63	0.15
Cd IV	0.16	0.16	0.32	0.10
Fungicide I	0.20	0.18	0.38	0.11
Fungicide II	0.16	0.16	0.32	0.10
Fungicide III	0.10	0.07	0.17	0.06
Fungicide IV	0.68	0.67	1.34	0.20

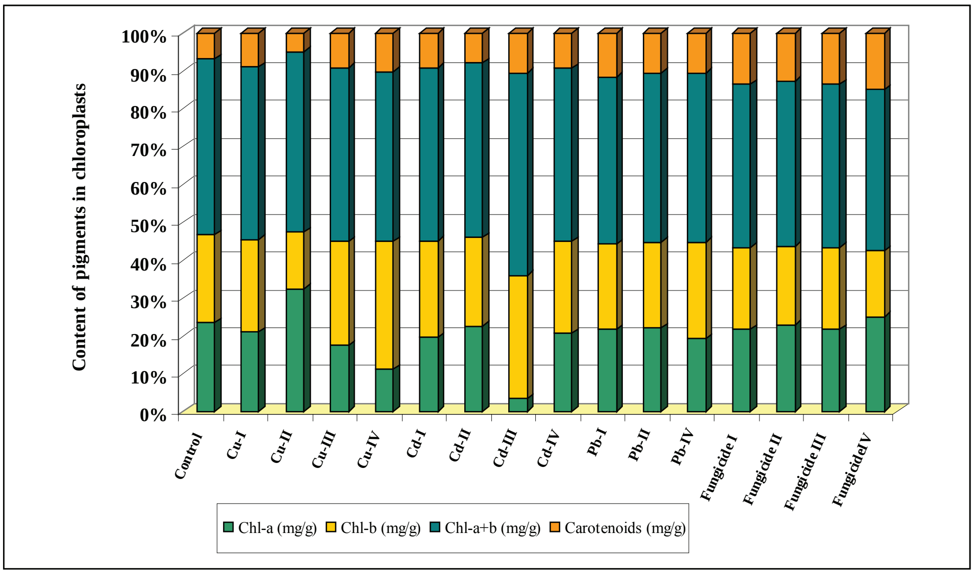

Figure 3. Content of pigments in chloroplasts in fresh material of tobacco leaves of variety Prilep P-156/1,upper belt (III incertion)

According to a number of literature references from internationally recognized laboratories, the process of photosynthesis can detect external contaminants. Chlorophyll assimilation, for example, can be used as highly relevant bio-indicator of toxic effects of many external contaminants.

Table 3. Content of chloroplast pigments (mg/g) in fresh material of tobacco leaves of variety Yaka Yv-125/3, upper belt (III incertion)

Treatment	Chl-a (mg/g)	Chl-b (mg/g)	Chl-a+b (mg/g)	Carotenoids (mg/g)
Control	0.33			0.14
Cu I	0.43	0.49	0.93	0.80
Cu II	0.38	0.30	0.68	0.14
Cu III	0.40	0.46	0.87	0.17
Cu IV	0.46	0.35	0.83	0.08
Pb I	0.44	0.38	0.83	0.08
Pb II	0.40	0.33	0.73	0.14
Pb III	0.31	0.32	0.63	0.11
Pb IV	0.22	0.20	0.42	0.09
Cd I	0.47	0.46	0.93	0.17
Cd II	0.38	0.45	0.83	0.15
Cd III	0.37	0.35	0.72	0.11
Cd IV	0.33	0.30	0.64	0.23
Fungicide I	0.28	0.28	0.55	0.11
Fungicide II	0.43	0.43	0.86	0.16
Fungicide III	0.22	0.23	0.45	0.09
Fungicide IV	0.40	0.48	0.89	0.17

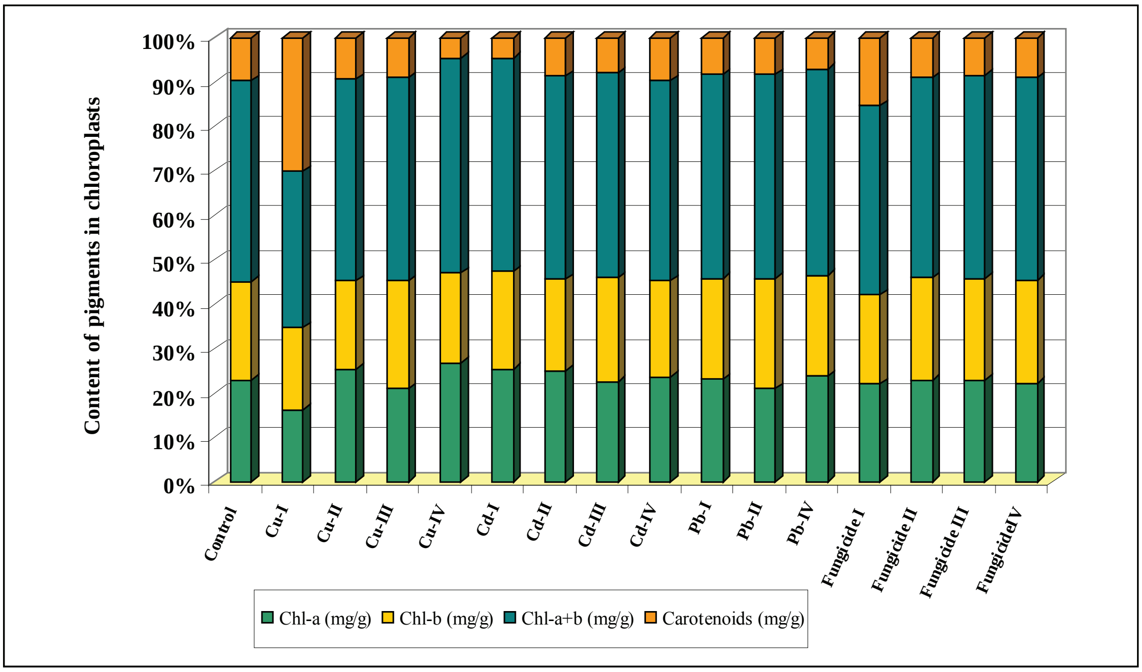

Figure 4. Content of pigments in chloroplasts in fresh material of tobacco leaves of variety Yaka Yv-125/3, upper belt (III incertion)

It can be stated from the results that the applied concentrations of heavy metals reduce the content of chloroplast pigments as a result of the chlorophyll destruction, but on the other side, the concentration of carotenoids is increased. High cadmium concentrations provoke disbalance and damages of the whole pigment system, which is reflected in reduced biosynthesis and organic production, In Prilep tobacco, chl-a decreases from 0.68 mg/g (control) to 0.05 mg/g (Cd

III concentration) and in Yaka tobacco, chl-b decreases from 0.32 mg/g (control) to 0.20 mg/g (Cd IV concentration). Copper is constituent part of the enzymes and thereby affects the life processes in plants. In plants treated with Cu, the chloroplast pigments content is slightly increased due to the general physiological effects of Cu accumulation in strengthening the structure of chloroplasts and in formation of organic matter. Chl-a concentration increases in both investigated types, ranging from 0.68 mg/g (control) to 0.90 mg/g (Cu II concentration) in Prilep and from 0.33 mg/g to 0.46 mg/g (IV concentration) in Yaka. Known by their constancy and protective function in chlorophyll stabilization, carotenoides are not significantly affected by application of heavy metals. The highest impact on their level was noticed after Pb application in soil (0.14 mg/g - 0.23 mg/g IV Pb).

According to investigations on poppy (Spasenoski M, Gadzovska S. 2000-2001), various concentrations of cadmium and lead affect the content of chloroplast pigments. Thus, the increased cadmium concentration reduces the content of these pigments. The results are in accordance with Skorzynska-Polit and Baszynski (1995), who report that cadmium causes significant changes of thylakoid membrane, which is in direct correlation with ultrastructural changes of the photosynthetic apparatus. Heavy metals and the fungicide are considered to have a destructive effect on biosynthetic apparatus, leading to disorder of the electron transport in one of the stages and in the process of formation of pigment system. As it is well known, tobacco (*N. tabacum*) can easily accumulate certain metals and particularly cadmium in leaves. Cadmium is indispensable, potentially toxic, widely spread contaminant, accumulated by tobacco and transferred through the smoke to humans.

Phosphate fertilizers applied in tobacco cultivation contain high concentration of heavy metals. A number of factors affect the amounts of heavy metals in tobacco: soil type, pH genotype, stalk position, soil and leaf residues resulting from metal-containing pesticides (Golia et co., 2001) and from soil amendments with fertilizers and municipal sludge.

Anthocyanins are powerful antioxidants which signalize the toxic effects of environment on plants. Together with certain enzymes, they are one of the first biomolecules which reveal the level of plant intoxication by changing their concentrations. The increase in heavy metals concentration in treated plants is a stress situation, but plants are attempting to normalize that situation (Table 4).

Table 4. Content of anthocyanins in dry tobacco leaves of varieties Prilep P-156/1 and Yaka Yv-125/3, upper belt (III incertion)

Treatment	Content of anthocyanins Prilep P-156/1 (mg/100 g)	Content of anthocyanins Yaka Yv-125/3 (mg/100 g)
Control	1.35	1.09
Cu I	1.02	1.47
Cu II	0.26	0.93
Cu III	0.56	0.34
Cu IV	1.02	0.69
Pb I	0.63	1.96
Pb II	0.29	0.46
Pb III	0.24	0.66
Pb IV	0.22	0.64
Cd I	0.49	0.46
Cd II	0.9	0.43
Cd III	0.25	0.51
Cd IV	0.49	0.17
Fungicide I	1.21	0.45
Fungicide II	0.64	0.85
Fungicide III	1.06	0.21
Fungicide IV	1.96	1.08

Although the two tobacco varieties investigated have similar physiological parameters, they differ by their resistance ability. Cadmium is potentially toxic and when its concentration is increased, it is included in the anthocyanins synthesis in treated plants. In our investigations, P-156/1 showed the highest anthocyanin contents with application of 1st, 2nd and 4th concentration of fungicide (0.2 mg/kg, 0.4 mg/kg and 1.6 mg/kg). In Yv-125/3 its highest value was recorded with application of 1st and 4th concentration of cadmium (0.1 mg/kg and 0.8 mg/kg), and 1st and 4th concentration of fungicide (0.2 mg/kg and 1.6 mg/kg). Direct fungicide application on tobacco leaves resulted in immediate antioxidant response, which leads to increased concentration of anthocyanins. Herefrom it can be concluded that Yaka tobacco has higher affinity to take protective measures in unfavorable conditions.

CONCLUSIONS

The study confirmed our expectations for the negative effects of heavy metals and disorder they make in the production of organic biomolecules. The following conclusions can be drawn from the results obtained:

1. Tobacco treatment with heavy metals Cu, Cd and Pb reduces the catalase activity.

2. The antioxidant enzyme increases its activity with the fourth concentration of fungicide in variety P-156/1 (1.96 mg dissolved H_2O_2), while in Yv-125/3 with the first concentration of copper (1.47 mg dissolved H_2O_2) and first concentration of lead (1.96 mg dissolved H_2O_2). In all other tobaccos treated with heavy metals, the levels of catalase activity were lower compared to the control variant.

3. High concentrations of cadmium cause disbalance and damage the whole pigment system, which is reflected in reduced biosynthesis. In Prilep tobacco, chl-a decreases from 0.68 mg/g (control variant) to 0.05 mg/g (Cd - III concentration). In Yaka tobacco, chl-b decreases from 0.32 mg/g (control variant) to 0.20 mg/g (Cd - IV concentration).

4. Copper increases the concentration of chloroplast pigments because it can be involved in strengthening the chloroplast structure.

5. Increased levels of anthocyanins in treated plants present a stress situation, but plants are attempting to normalize the stress.

6. Stress situation in P-156/1was noticed with first, second and fourth concentration of fungicide (0.2 mg/kg, 0.4 mg/kg and 1.6 mg/kg). In Yv-125/3 it was recorded with first and fourth concentration of cadmium (0.1 mg/kg and 0.8 mg/kg) and first and fourth concentration of fungicide (0.2 mg/kg and 1.6 mg/kg), which resulted in increased anthocyanins concentration.

7. Direct fungicide application on tobacco leaves resulted in an immediate antioxidant response, resulting in increasing the anthocyanins concentration.

8. Herefrom it follows that Yaka tobacco is more capable of taking protecting measures in unfavorable conditions.

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