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Heavy Metals and 2,4-Dinitrophenol Impact on Some Physiological and Biochemical Parameters in *Capsicum* Species

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HIGHLIGHTS

- DNP and heavy metals' salts affected viability of *Capsicum* species.
- The activity of antioxidant enzymes varied significantly with the applied treatment.
- The presence of pesticides and heavy metals in the soil represents a major risk for the environment.

Abstract: The accumulation of heavy metals and of pesticides in the soil have a negative impact on *Capsicum chinense* var. *Aji Mochero* and *Capsicum baccatum* var. *pendulum Bishop's Crown* species, the present work highlighting the exerted influence on the superoxid-dismutase activity, catalase and peroxidase – as enzymes implied in the removal of H_2O_2, of O^{2-} radical and of ion HO^- that have devastating effects on the vegetable cell, on the Krebs cycle's dehydrogenases – as main way to produce energy, respectively of the chlorophyll *a*, *b* and carotenes – as photoassimilatory pigments, but also as biologic antioxidants.

Keywords: dinitrophenol; heavy metals; *Capsicum*; oxidative stress.

INTRODUCTION

The presence in the environment of the pesticides and of the different heavy metals turns on serious problems due to their toxicity significantly high and to the fact that vegetable organisms have the capacity to absorb from the soil these substances through roots and from the air, with the help of foliar apparatus, from where there are translocated at the level of the hole organism and from here on appears the food chain, representing an enormous risk for human's health and for animals [1].The specialty literature indicates the ecological

risk's potential of peppers contamination with Fe, Cd, Pb and Ni, risk as higher as in the soil and the aerial parts of the *Capscium* gender there is a Mn deficit [2].

The *Capsicum* species gender contain a large variety of nutritional compounds, also active pharmacologic metabolites [3], being frequently used for different digestive problems, circulatory, neuropathy associated with diabetes mellitus and HIV, rheumatoid disease, sinusitis and different migraines [4].

The present work aimed to evaluate the biochemical and physiological answer of *Capsicum chinense* var. *Aji Mochero* and *Capsicum baccatum* var. *pendulum Bishop's Crown*, species, cropped in lab conditions, at the action of some pesticides and salts of heavy metals taking a common sense view of superoxid-dismutase activity (SOD), catalase (CAT), peroxidase (POX), Krebs cycle's dehydrogenases (izocitrat-dehydrogenase – IDH, ketoglutarat-dehydrogenase – KDH, succinat-dehydrogenase – SDH and malate-dehydrogenase – MDH) and of the content of chlorophyllien and carotenoidic pigments.

MATERIAL AND METHODS

Plant material

The researches were done on two types belonging to *Capsicum* gender namely *Capsicum chinense* var. *Aji Mochero* and *Capsicum baccatum* var. *pendulum Bishop's Crown* cropped in lab conditions. Thus, the seeds were cropped in vases of 25 cm diameter, at 3 cm deepness in the soil and moistened, at three days, during five weeks, with constantly volume of distilled water, obtained through a MilliQ (18,2 Ω) system. Subsequently was resorted to the apply of different types of treatments [$(CH_3COO)_2Pb$, $FeSO_4$, $SnCl_2$], the solutions used being prepared so that the final concentration of the metal to be of 0.005 M. In parallel we resorted at the obtaining of some samples treated with 2,4 – dinitrophenol (DNP), in concentration of 0.005M, substance that enters in composition of different pesticides, weed killers, fungicides, ovicides, acaricides etc. and thus used more frequently in the last years, in the modern agricultural practices. For each type of treatment in part were obtained three parallel plots of peppers, the experimental results representing the average of these repetitions ± the standard deviation. The harvest of the samples was done at two months after the beginning of applying treatments.

Reagents and instruments

Were used: TRIS-HCl 0.1M with pH 7.8 buffer solution which contain 1 mmol dithiothreitol and 1 mmol EDTA; buffer solution potassium phosphate 0.067M with pH 7.8, 0.01M with pH 7.0 and, respectively, 0.4M with pH 5.9; EDTA disodic 0.01M with pH 7.8; Nitro Blue Tetrazolium (NBT) 1.5mM solution; riboflavine solution 0.12mM; potassium bicromate 5% in glacial acetic acid ; o-dianisidine 1% in ethylic alcohol 96%; H_2SO_4 50%; H_2O_2; acetone; Coomassie Briliant Blue G_{250}.

All the reagents used were of high analytic purity, and the solutions were prepared in distilled water obtained in a MilliQ system. The extinctions determination was done with a UV-VIS Shimadzu 1700 spectrophotometer, in quartz cuves of 1 cm, towards a control of the reagents, specific to each method in part.

Principle of the methods

SOD (superoxide: superoxid - oxidoreductase, EC 1.15.1.1) catalyze the dismutation of the free radical superoxide in O_2 and H_2O_2, its activity being determined on the enzyme's capacity to inhibit the NBT reduction of superoxide radicals generated in the reaction medium through riboflavin's photoreduction. The inhibition degree produced by the enzyme, in standard conditions, was estimated through the determination of samples' and controls' extinctions at 560 nm, towards the distilled water [5].

CAT (H_2O_2: H_2O_2 – oxidoreductase, EC 1.11.1.6) is implied in the detoxification of the hydrogen peroxide, species oxygen reactive which results, after different ordinary and

pathologic processes, under the SOD action and of the different oxidases. The activity of this enzyme was estimated by Synha method, colorimetric determination based of the chromic acetate obtained by reaction of the potassium bicromate's reduction in the acid medium by the distilled water left-over undissolved after the enzyme's inactivation, at 570 nm [6].

POX (donor: hydrogen peroxide – oxidoreductase, EC 1.11.1.7) performs a significant role in the oxido-reducing processes correlated with the plants respiration, the enzyme being able to oxidize, in H_2O_2 presence a big number of substratum like fenols of pirocatechine's type, pirogalol and galic acid, the aromatic amines such as benzidine and p-phenilendiamine, but also substances slightly oxidable of vitamin C and glutathione type. The method of this peroxidase's activity dosage is based on measuring the oxidation product color's intensity of o-dianisidine with H_2O_2 help, the formed product having a maximum absorption at the 540 nm wavelength [5].

The concentration of total soluble proteins was determined through Bradford method which is based on the observation that, in acid medium, the Coomassie Brilian Blue G_{250} reagent reactions with radicals of arginine, histidine, lizine, tirozine, triptophane and phenilalanine from the proteins' structure, the formed complex having a maximum of absorption at 595 nm [7].

The content of chlorophyllien pigments which represent the prosthetic groups of the chlorophylls and those carotenoidic was estimated on spectrophotometrical way, after the extraction with acetone, at 663, 645 and 472 nm wavelengths [8].

Statistics

The statistic processing of the experimental data obtained was realized with the SPSS20 program (Statistical Package for the Social Sciences, Chicago, Illinois) and Microsoft Excel (the t-Student test), being considered significant at values of p littler than 0.05. The data were expressed as medium values on each plot ± standard deviation.

RESULTS AND DISCUSSION

The environment's pollution with heavy metals, as a result of different industrial activities, but also with a series of pesticides, fungicides, ovicides etc. used in the agricultural maintenance techniques, has a negative impact on the human's health and animals' [9-12]. Although the negative effects due to different toxic substances are knew long time ago, they continue to be used on large scale in the whole world, the pollution with heavy metals and pesticides being a major problem in lots of geographical areas [13]. The specialty literature signalize the importance of the interactions between the synergic circumstances and antagonists based, especially, on the individual effects of each pollutant in part, imposing the necessity to know the impact produced by the combinations of metallic ions in different concentrations at the plants' level [14]. Furthermore, the development and the productivity of plants are profoundly affected by the environment's pollution, the different biotic and abiotic factors producing major imbalances at the cellular level, the resulted stress being accompanied by the storage of an enhanced quantity of reactive oxygen species [15, 16].

The apply of DNP treatments and salts of heavy metals at the *Capsicum chinense* var. *Aji Mochero* species had different consequences on the activity of SOD, CAT and POX, in the sense that, for the most part, leaded to significant increases of the activities of these oxido-reducing enzymes, strongly correlated with the concentration of the formed free radicals (Figure 1). Thus, if at the control, the SOD activity reach a valoric threshold equal with 50.221±1.265 EU/µg protein, at the experimented variants treated with DNP and $SnCl_2$ solutions the enzyme registers maximal values of approximately 63 EU/µg protein, while the plots treated with $(CH_3COO)_2Pb$ and $FeSO_4$ remark their self by an inhibition degree more diminished of the NBT reduction by the superoxide radicals generated in the reaction medium through riboflavine's photoreduction (36.426±1.009 EU/µg protein, respectively 41.221±1.156 EU/µg protein). An enzymatic unity is defined as being equal with amount of enzyme required to cause 50% inhibition of the NBT reduction.

The superoxide radicals are comparatively less toxic but are highly reactive and leads to a series of chain reactions thus generating very toxic radicals via Haber Weiss reaction [17]. The specialty literature signalize the fact that the metals can be essential and non-essential for the plants' growth, with different answers in function of the concentration for both classes, some of them having a crucial role for the vegetable organisms' metabolism, while others, even at reduced concentrations, produce imbalances at the level of biochemical and physiological processes with repercussions on the plants' growth and productivity [18-20]. On the other hand, ROS and H_2O_2 especially, are considered essential compounds of the transgoing signal used by the plants to answer to the developmental and environmental cues [21], SOD being the first defense line in the antioxidant defensive [22].

The CAT activity oscillates in largely limits, the medium value being decelated at the control plot (18.312±0.659 EU/µg protein), and the maximum in the case of the treatment with $(CH_3COO)_2Pb$ (44,802±1.025 EU/µg protein) and DNP (42.227±1.235 EU/µg protein), while POX lays out a maximum valoric threshold of 12.459±0.556 EU/µg protein in the case of the sample treated with DNP, followed by that one treated with $FeSO_4$ (9.726±0.065 EU/µg protein) and $(CH_3COO)_2Pb$ (7.565±0.12 EU/µg protein). To mention that a catalasic unity represents the enzyme quantity which decompose a micromole of H_2O_2 during 1 minute at pH 7.0 and 20ºC temperature, while a peroxidase unity corresponds to the enzyme's quantity which catalyze the decomposing of a micromole H_2O_2 in a minute in optimum conditions.

A series of researches demonstrated the phytotoxicity particularly high of the DNP which produce major imbalances of the metabolic reactions as a consequence of the free oxygen radicals' accumulation, the increase of oxidative stress' level determining implicitly the increase of oxido-reducing's activity [23, 24]. In the same time, the iron excess [25] and lead [26] in plants may lead to the leaves' necrosis as a sign of the oxidative stress' installment, the storage of the lead at cellular level having as non-specific visual symptoms, the inhibition of roots growth, the stagnation of plant growth, or even the cellular death [27, 28].

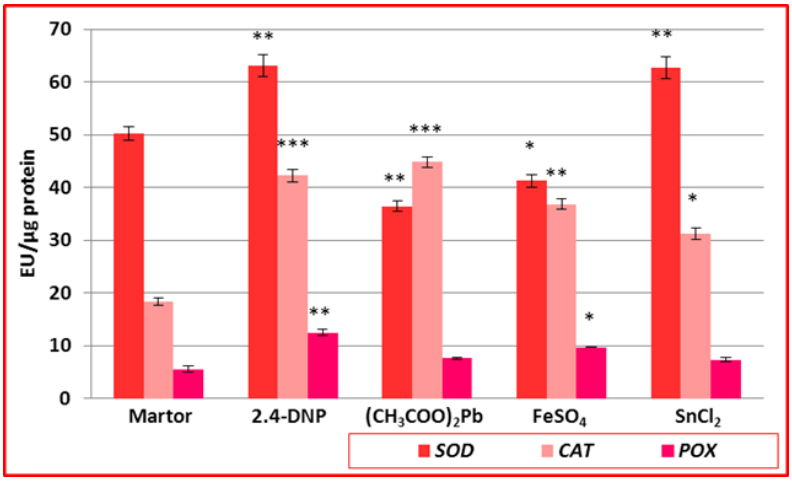

Figure 1. SOD, CAT, POX activity at *Capsicum chinense* var. *Aji Mochero* species (*p < 0.05 – less significant;**p < 0.01 – significant; ***p < 0.001 – very significant).

In what concerns the *Capsicum baccatum* var. *pendulum Bishop's Crown* species, the SOD, CAT, POX activity present a similar manner of behavior, being unremarkable notable differences comparatively with *Capsicum chinense* var. *Aji Mochero* species (Figure 2). Thus, at SOD, the activity presents largely limits of variation, in the sense that the apply of DNP treatment leaded to an intensify of the superoxide radicals' mobilization comparatively with the reference plot (62.892±1.965 EU/µg protein towards 41.599±1.126 EU/µg protein), while, also this time, $(CH_3COO)_2Pb$ inhibited the NBT reduction, but in a diminished percent (36.693±1.11 EU/µg protein). Besides, the scientific literature highlights the decreasing of some enzymes' activity at different lead concentrations in the environment [26].

The CAT activity was intensive stimulated by DNP used during the experiment, the enzyme registering a medium value of approximately three times higher comparatively with the moistened plot with water (46.465±1.023 EU/µg protein, respectively 16.268±0.569 EU/µg protein), while $SnCl_2$ and $FeSO_4$ were highlighted their self by moderate effects, the enzyme laying out values of approximately 25 EU/µg protein.

POX is an enzyme implied in multiple cellular processes like the polymerization of aromatic monomers, lignification, the cells' elongation, auxines' catabolism, the phenolic oxidation and the defense against the biotic and abiotic stress represented by different pathogens [29-32].

Also in this case, the apply of the DNP treatment had as consequence the increase of POX activity (14.435±0.653 EU/µg protein in the treated plot comparatively with 9.725±0.336 EU/µg protein at the reference plot), demonstrating, once again, the high toxicity of this agent. The $(CH_3COO)_2Pb$ and $FeSO_4$ solutions had approximately the same impact in what concerns the storage of ROS, the enzyme laying out the medium value of 10.25 EU/µg protein, while the treatment with $SnCl_2$ was remarked through an inhibition of the enzymatic activity, the medium value registered being with approximately two unities littler by comparison with the plot treated with water (7.324±0.62 EU/µg protein).

In explaining the results we must take into account that the modify of the activity of this oxidoreductase oscillates in strong connection with the plants' age and the duration of the treatment administered [33], the specialty literature signalizing a capacity of peroxidation different function of the stage in which there are the processes of cellular differentiation and development of the tissues [34-36].

The statistical analyze regarding the activity of some enzymes implied in the oxidative stress highlighted, at both species, the existence of some significant and strongly significant differences between the reference plot and the samples treated with DNP and $(CH_3COO)_2Pb$ in what concerns the SOD and CAT activity ($0.01 < p < 0.001$).

The experimental data obtained concord with those from the scientific literature which signalize the stimulatory influence that $FeSO_4$ exerts, respectively the Fe ion from different other salts, on the POX's activity [37, 38]. Other studies highlight the iron's importance in quality of main cofactor of many other antioxidant enzymes, this element being able to function as a prooxidant forasmuch catalyzes the free radicals by Fenton reaction [39], its direct influence on CAT and SOD activity depending on its concentration in the environment, the increase activity of SOD being able to appear as a result of the superoxide radicals' storage at the leaves' level, which or is suffering of iron deficit [40], or they are having a redundancy of this metallic ion [41]. On the other hand, comparatively with the control plot, the Pb treatment may cause the significant increase of CAT and POX activity from the foliar tissue, in parallel with the reduction of increasing rate of the root and of the stem, but also of the chlorophyllien pigments content [42].

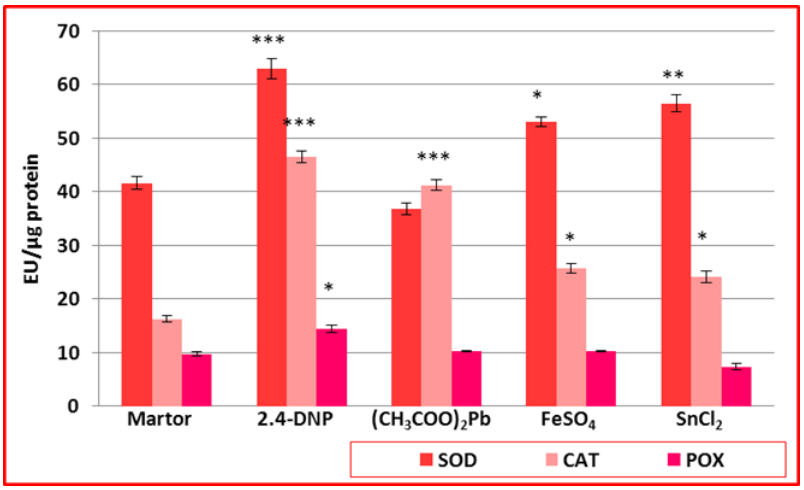

Figure 2. SOD, CAT, POX activity at *Capsicum baccatum* var. *pendulum Bishop's Crown* species (*p < 0.05 – less significant;**p < 0.01 – significant; ***p < 0.001 – very significant).

In the same time, was demonstrated the fact that the CAT activity, in the lead presence, is lower in roots and more significantly at the stem's level and at the foliar device, and it depends of the heavy metal's concentration, up to a certain concentration, considered moderate-toxic, the enzymatic activity increasing significantly, the plants presenting high tolerance towards this ion, while, at higher concentrations, strongly toxic, the activity decreases drastically, and the effect on the vegetable organisms is devastating [43].

Another purpose of our study was the evaluation of Krebs cycle's dehydrogenases' activity under the influence of the above treatments. At the control plots, in the case of *Capsicum chinense* var. *Aji Mochero* species (Figure 3), the dehydrogenasic activity varied between 10.956±0.35 µg formazan/g fresh tissue at IDH and 5.525±0.3 µg formazan/g fresh tissue at KDH, while, the different treatments applied had effects more or less significant and different from an enzyme to another. Thus, IDH registered the maximum valoric threshold at the plot treated with $(CH_3COO)_2Pb$ (24.841±0.3 µg formazan/g fresh tissue), followed by 2,4 – DNP (19.282±0.08 µg formazan/g fresh tissue) and $FeSO_4$ (15.349±0.55 µg formazan/g fresh tissue), while KDH and SDH were significantly stimulated by $SnCl_2$ (8.516±0.025 µg formazan/g fresh tissue) and $FeSO_4$ (9.223±0.45 µg formazan/g fresh tissue). The malate's mobilization takes place with a littler rate, being ascertained medium values net inferior to control in the case of DNP, $FeSO_4$ and $SnCl_2$, at the variant treated with Pb the enzyme reaching valoric levels comparative to the reference sample (9.215±0.35 µg formazan/g fresh tissue).

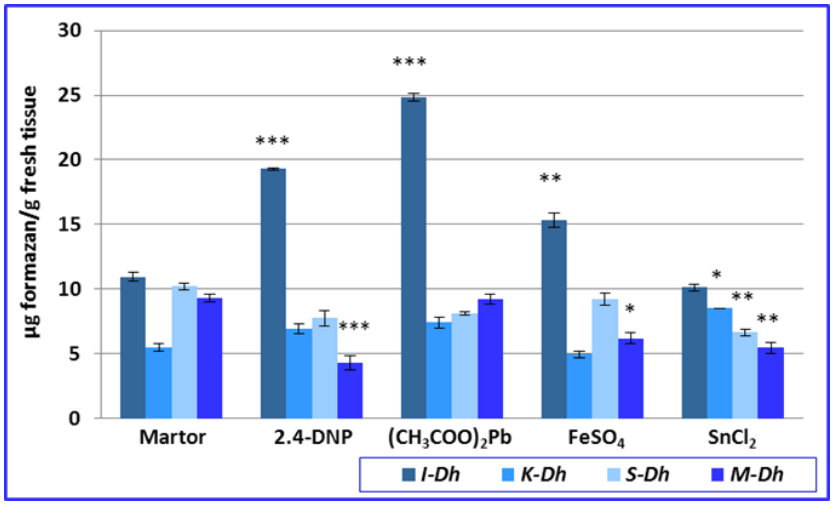

Figure 3. Krebs cycle dehydrogenases activity at *Capsicum chinense* var. *Aji Mochero* species (*p < 0.05 – less significant;**p < 0.01 – significant; ***p < 0.001 – very significant).

At *Capsicum baccatum* var. *pendulum Bishop's Crown* (Figure 4) can be observed medium values generally decreased, IDH laying out activities contained in the interval 6.942±0.25 µg formazan/g fresh tissue (in the case of applying treatment with Pb) and 9.971±0.28 µg formazan/g fresh tissue at the reference sample, $FeSO_4$ and $SnCl_2$, inhibiting equally the activity of these oxidoreductase (approximately 7.5 µg formazan/g fresh tissue on each plot). The α-ketoglutarate in succinil-CoA conversion takes place in a different manner in the studied plots, the KDH activity reaching the medium value of 8.231±0.2 µg formazan/g fresh tissue in the case Pb acetate, 9.326±0.35 µg formazan/g fresh tissue at $FeSO_4$, approximately 10 µg formazan/g fresh tissue at the applying of DNP and $SnCl_2$ solutions and 15.693±0.25 µg formazan/g fresh tissue at the control plot. SDH and MDH were stimulated by the agents used, maximal values being met in the case of the variants with $SnCl_2$ (9.526±0.25 µg formazan/g fresh tissue for SDH and 6.412±0.35 µg formazan/g fresh tissue for MDH). It must be underlined the high importance in evaluating the oxidoreductases' activity of the biologic material's harvest moment and the stage in which there is overtook the Krebs cycle, the specialty literature underlying the IDH implying in the cellular senescent process, the enzyme having a direct role in storing with NADH necessary for the antioxidant protection of the mitochondria [44]. On the other hand, it is to remember

the fact that, in addition to the formed compounds in the Krebs cycle and degraded in a subsequently stage of this one, the substratum specific to the enzymes in the case, may derive also on other anabolic or catabolic ways [45].

The statistical processing of the results regarding the dehydrogenases' activity's Krebs cycle, points the existence of some differences strongly significant ($p<0.001$) between the reference samples and that treated with DNP and Pb acetate at the *Capsicum chinense* var. *Aji Mochero* species (in the IDH's case), respectively significant ($p<0.01$) and strongly significant ($p<0.001$) in the case of applying Pb, Fe and Sn, on the activity KDH and SDH, at the *Capsicum baccatum* var. *pendulum Bishop's Crown* species.

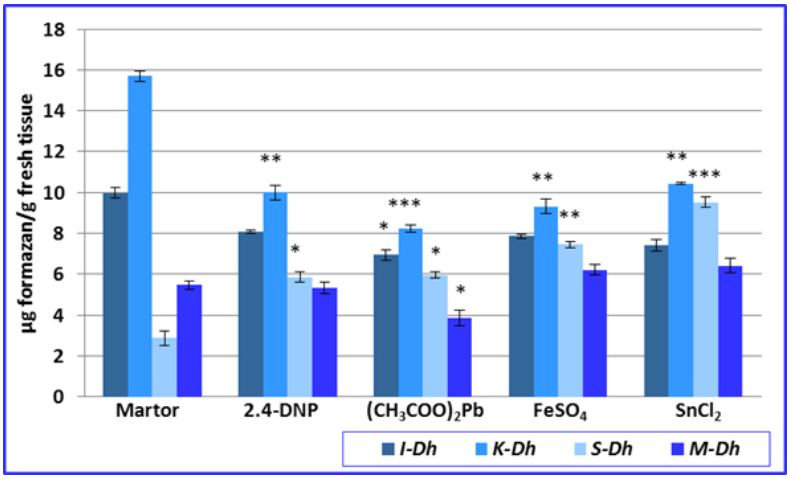

Figure 4. Krebs cycle dehydrogenase activity at *Capsicum baccatum* var. *pendulum Bishop's Crown* (*p < 0.05 – less significant;**p < 0.01 – significant;***p < 0.001 – very significant).

Being known the fact that the heavy metals have a strong impact on the enzymes' activity implied in the antioxidant defensive on the one hand, but also on the photosynthesis' and sweating rate, respectively on the chlorophyll and carotenes content, on the other hand [46], in a last series of experiments we had recourse to evaluating the physiologic answer of the two species of peppers at the agents' action input in the growing environment taking a common sense view of the concentration of chlorophyllien and carotenoidic pigments. Besides, the answer and the accommodation of the plants to continuous fluctuations of the environment's conditions are translated by physiological and biochemical modifications to survive in different stress conditions [15].

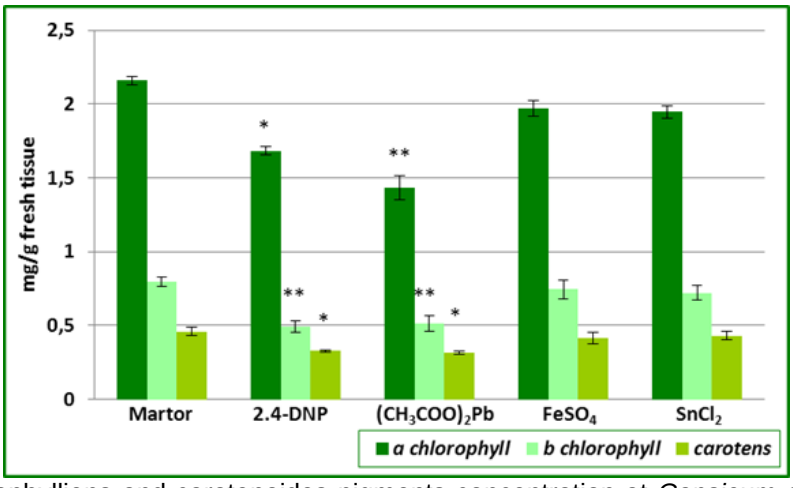

Figure 5. Chlorophylliens and carotenoides pigments concentration at *Capsicum chinense* var. *Aji Mochero* species (*p < 0.05 – less significant;**p < 0.01 – significant; ***p < 0.001 – very significant).

In what concerns the chlorophyll a, photosynthetic pigment which helps the plants to procure itself energy from the light, we can ascertain a slightly diminution of the content comparatively with the reference plot (2.158±0.025 mg/g fresh tissue), the medium values

decelated being of 1.432±0.08 mg/g fresh tissue at the variant containing Pb acetate, 1.683±0.03 mg/g fresh tissue at that with DNP and approximately 1.95 mg/g fresh tissue at the plots treated with Sn and Fe (Figure 5). By comparison, the chlorophyll b and the carotenoids lay out values much more decreased, being ascertained, nevertheless, significant differences between the control plot and the experimental variants in which were applied solutions of DNP and $(CH_3COO)_2Pb$ [0.796±0,03 mg/g fresh tissue - control, 0.492±0.04 mg/g fresh tissue – DNP and 0.512±0.055 mg/g fresh tissue - $(CH_3COO)_2Pb$ for the chlorophyll b, respectively 0.46±0.03 mg/g fresh tissue - reference carotenes and approximately 0.32 mg/g fresh tissue at DNP and $(CH_3COO)_2Pb$].

At the *Capsicum baccatum* var. *pendulum Bishop's Crown* species the situation is slightly different, in the sense that, at the plots treated, there are ascertained valoric thresholds slightly superior of the chlorophyll a and b comparatively with the control sample, excepting the variant with $FeSO_4$, where the decelated concentration was under the reference threshold (1.515±0.0035 mg/g fresh tissue and 1.407±0.055 mg/g fresh tissue – chlorophyll a, respectively 0.634±0.03 mg/g fresh tissue and 0.58±0.04 mg/g fresh tissue – chlorophyll b). The specialty literature highlights the influence that exerts the different concentrations of the pesticides [10, 47] and of the heavy metals [48-50] on the assimilatory pigments content. Different studies [51-53] signalize the reducing of the chlorophyll's a and b level and of the carotenes under the influence of Fe and Pb ion's which can be associated with the absorption and the deficitary distribution of the essential nutrients, which has as consequence the growing process' perturbation of the plants.

The carotenes content, as biologic antioxidants and key factors in reducing the diseases incidence, it's remarked through medium levels slightly decreased, highlighting values of the order of 0.371±0.01 mg/g fresh tissue at the variant with DNP, 0.337±0.04 mg/g fresh tissue at that with $FeSO_4$ and 0.357±0.03 mg/g fresh tissue at the plot grew on the environment containing $SnCl_2$. Significant differences between the control plot and the variant with Pb were remarked after applying the test of statistical signification Student, the carotenes' concentration in the two plots being of 0.426±0.03 mg/g fresh tissue, respectively 0.256±0.04 mg/g fresh tissue (Figure 6).

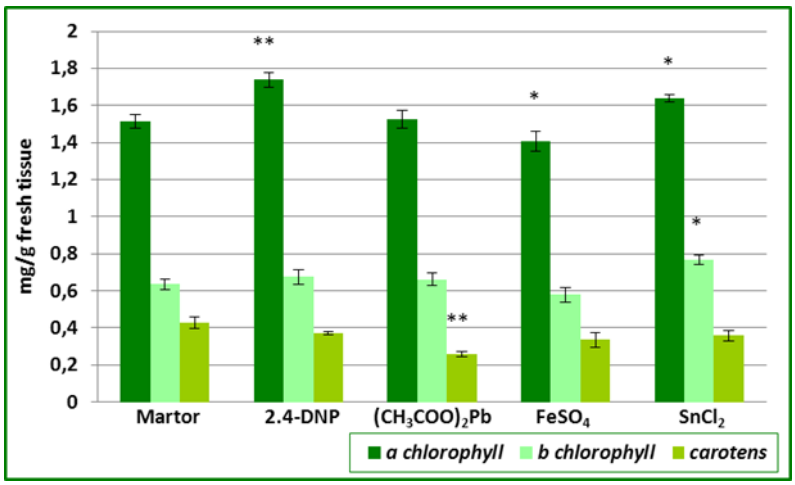

Figure 6. Chlorophylliens and carotenoides pigments concentration at *Capsicum baccatum* var. *pendulum Bishop's Crown* species (*p < 0.05 – less significant;**p < 0.01 – significant; ***p < 0.001 – very significant).

The carotenes concentrations, according to recent studies [54-56], it's influenced by a series of factors like the presence of different biotic and abiotic factors in the environment, the genotype, the agronomic factors etc.

CONCLUSION

Our results demonstrate the impact deeply negative of the applying pesticides and heavy metals' salts on the *Capsicum chinense* var. *Aji Mochero* and *Capsicum baccatum* var. *pendulum Bishop's Crown* species' viability, the SOD, CAT, POX activity and Krebs

cycle's dehydrogenases, as well as the concentration of chlorophyllien pigments and carotenoids being significantly influenced by the presence of the respective agents in the growth environment, strongly correlated with the level of oxidative stress generated by the quantity of ROS produced in the cell. Taken together, these results suggest the necessity of fixing some dosages rigorously determined by the pesticides and other agents with recognized phytotoxicity, with the aim of controlling the physiological and the biochemical answer of the plants at the storage of free oxygen radicals, the vegetable organisms having the capacity of adapting to the presence of heavy metals in soil, the tolerance degree of these ones being assured by the presence of enzymatic and non-enzymatic equipments.

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