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EFFECT OF HEAVY METALS ON THE GERMINATION OF WHEAT SEEDS: ENZYMATIC ASSAY

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ABSTRACT

Stress caused by heavy metals is a major problem which affects agricultural productivity and, implicitly, human health. Natural flora presents differences of tolerance to heavy metals. Some plants grow well in a soil enriched with heavy metals, while others cannot develop in such conditions. This study investigates the effect of heavy metals on plant viability at molecular level and draws attention to the danger of the widespread use of toxic compounds.

KEYWORDS: heavy metals; genetic conservation; toxicity; enzymatic determination; germination tests

1. Introduction

Plant viability can be partly and irreversibly degraded under the action of certain physical agents or chemical substances [1,2]. The phenomenon can occur under the action of metabolic inhibitors, heat, electricity, UV radiation, or it can occur spontaneously or in different physiological, pathological and experimental conditions [3]. Therefore, the state of living matter should be looked at as being dependent on the state of biostructural matter [4, 5]. Namely, living organisms being affected can signify physiological and even morphological modifications.

The toxicity of heavy metals that enter vegetal tissues can inhibit multiple physiological processes, such as: growth factors, photosynthesis, water absorption and nitrates assimilation [6, 10]. The gravity of these effects largely depends on the concentration of ions in heavy metals and the sensitivity or tolerance of plants affected by their presence [11, 12]. Some metals in small concentrations (Co, Cr, Cu, Fe, Mn, Mo, Ni, V and Zn) are considered essential to plant growth [13]. Zn and Co, as well as Mn and Fe, are essential elements [14] to the superior part of plants and are involved in more metabolic processes, while Pb and Cd do not have any physiological function in plants. A deficit of Zn and Co determines modifications in the fundamental processes of plant metabolism, which leads to a reduced growth. Root growth is more sensitive to heavy metal contamination [15, 16]. An immediate effect of plant contamination with a high concentration of Co is the side roots growth inhibition; these stay shorter, very ramified and without a solid structure. The experiment proved the fact that treating wheat seeds with heavy metals leads to a biomass reduction [15]. Consequently, the leaf function can be directly affected by local accumulation of heavy metals, or indirectly, by affecting the roots [17].

Nosko *et al.*, 1988 signals the fact that using Al in various concentrations does not inhibit the *Picea glauca* seed germination rate, modifying their viability on the other hand [18]. Peralta *et al.*, 2001 shows that different concentrations of Cr, Cu, Cd, Ni and Zn reduce the germination rate of *Medicago sativa L.* seeds and the elongation of rootlets and stemlets of future plants [19].

Munzuroglu and Geckil, 2002, after studying the influence of heavy metals Hg, Cd, Co, Cu, Pb and Zn upon the germination of *Triticum aestivum* și *Cucumis sativum* seeds, highlight a different inhibition of this process based on the used

concentration, as well as a reduction of the rootlet, hypocotyl and coleoptile length [20].

In our research, we emphasized the toxic effect on the viability of wheat seeds, using plants exposed to germination in the presence of heavy metal salts. Also, molecular modifications were highlighted through enzymatic measurements.

2. Materials and methods

In this study, we used analytical grade reagents, while all solutions were prepared with milliQ grade water with R = 18.2 Ω. Heavy metals were purchased from Sigma Aldrich (USA), Merck and Fluka.

Biological material. Wheat seeds (*Triticum Aastivum*), Gasprom variety, acquired from the Agricultural Research Station Suceava.

Instruments. Absorbance spectra were recorded with a Libbra S35 PC UV/VIS spectrophotometer endowed with quartz cuvettes with optical path length of 1 cm. Microprocessor Cole Parmer Ultrasonic (USA, Illinois) used for enzymatic extraction. Mikro22R centrifuge (Hettich) used for the separation of soluble protein mixture containing insoluble components (cell membrane). HANNA pH meter PH 211 used for determining and adjusting the pH buffers.

Germination determining. Lots of 50 seeds each were treated with different solutions of heavy metals inhibitors (solution 10^{-3} M salt of Cu^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Ba^{2+}, Ag^{+}, etc.), then left to germinate on filter paper in Petri dishes. Treatment duration is 1 hour, after which seeds are disposed in Petri dishes as uniformly as possible, on double filter paper, together with a treatment solution. Thus, 7-day wheat plantlets were harvested from their seeds, measured (height **H**, expressed as cm) and weighed (**M**, expressed as grams).

Enzymatic assay. The biochemical study of wheat seeds and plantlets consisted of determining the activity of some enzymes of oxidative stress [21]. Thus, to determine the peroxidase activity, we used the Gudkova and Degtiari, 1968 method (Artenie *et al.*, 2008), which is based on measuring the wave length of 540 nm of the color intensity of the oxidation product of o-Dianisidine with the help of peroxide. For the catalase activity, we used Sinha, 1972 method cited by Artenie *et al.*, 2008, by colorimetrically determining (λ = 570 nm) the chromic acetate obtained through the reaction of reduction of the potassium dichromate in acid environment by the peroxide which remained undecomposed after the enzyme inactivation. The superoxide dismutase activity was evaluated with the Winterbourn *et al.* (1975) method adapted by Artenie *et al.*, 2008, the method consisting of the enzyme capacity to inhibit the reduction of Nitro Blue Tetrazolium by the superoxide radicals generated in the reaction environment through riboflavin photoreduction (λ = 560 nm) [22].

The concentration of total soluble proteins was obtained based on the Bradford (1976) method, by forming a complex at an absorption maximum of 595 nm [23] between the proteins and the Coomassie Brilliant Blue G-250 dye.

3. Results and discussions

Germination test.

Fig. 1 shows that the effect of heavy metals at low concentrations (10^{-3} M) barely differs, manifesting a slightly inhibitory effect upon the germination of wheat seeds. As a result of this experiment, we can affirm that the toxic effect of heavy metals increases together with the concentration [24].

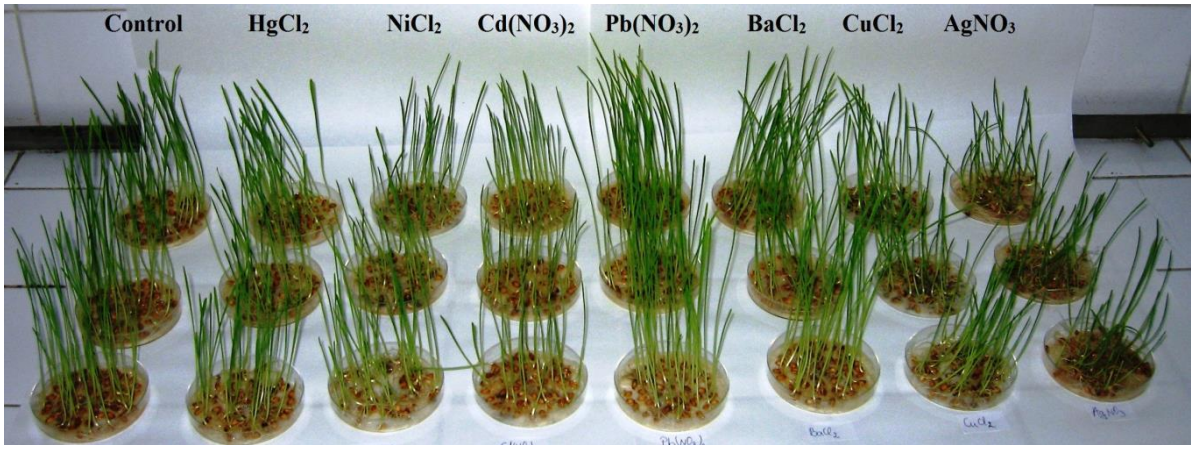

Fig. 1. *Effect of heavy metals (10^{-3}M) on wheat seeds after 7 days of germination*

After processing the obtained results (see Table 1), we noticed that plantlet length is similar in case of $HgCl_2$, $NiCl_2$, $Cd(NO_3)_2$, $CuCl_2$ and $AgNO_3$, but significantly differs from the blank sample, which leads to us affirming that these heavy metals inhibit the height growth of wheat plantlets. $Pb(NO_3)_2$ and $BaCl_2$ salts enhance the height growth of plantlets: from 409.50 cm in case of the blank sample, to 442.60 cm for $Pb(NO_3)_2$ and 434.83 cm for $BaCl_2$.

Tabel 1. *Effect of heavy metals ($10^{-3}M$) on wheat seeds*

Treatment *)	**No. plantlets**	**No. germinated seeds**	**No. dead seeds**	**Heights of plantlets in 50-seed lots (cm)**	**Plantlet weight of 50-seed lots (g)**
Control	34.60±0.20	1.30±0.10	14±0.12	2.54±0.33	409.50±2.54
$HgCl_2$	33.60±0.24	1.30±0.05	15±0.14	2.09±0.20	313.80±0.76
$NiCl_2$	35±0.43	0.30±0.10	14.60±0.98	2.02±0.36	302.20±0.50
$Cd(NO_3)_2$	37.30±0.15	0.60±0.01	15.30±0.25	1.94±0.05	302.80±5.78
$Pb(NO_3)_2$	35.30±0.23	0.30±0.02	17.60±0.45	2.40±0.10	442.60±3.64
$BaCl_2x2H_2O$	33.64±1.25	0.30±0.01	16±0.50	2.55±0.20	434.83±6.57
$CuCl_2$	35.30±0.50	1.30±0.25	13.30±0.75	2.17±0.37	314.30±5.73
$AgNO_3$	35±1	1.30±0.02	13.60±1.23	1.92±0.53	299.10±7.89
*) mean of three independent values					

The variation of enzymatic activities based on the treatment with heavy metals $10^{-3}M$.

Practical study of the superoxide dismutase (SOD) activity from vegetal extracts.

The data in Table 2 shows that the activity of this enzyme stabilizes the cellular abilities of removing oxygen radicals and attenuating cellular damage. Therefore, compared to the plantlets in the control batch, where the average activity of the SOD was 5.43 USOD/mg protein, the level of SOD activity increased significantly up to 14.36 USOD/mg protein in case of seeds treated with $NiCl_2$. The increase of oxidoreductase activity in the experimental group can be consequently attributed to the intensification of processes that release superoxide radicals under the initial influence of heavy metals.

Table 2. *SOD values in wheat plantlets after 7 days of treatment*

Treatment	**USOD/mg protein**
Control (distilled water)	5.43
$HgCl_2$	8.96
$NiCl_2$	14.36
$Cd(NO_3)_2$	10.21
$Pb(NO_3)_2$	7.87
$BaCl_2$	4.98
$CuCl_2$	9.51
$AgNO_3$	9.24

Quantitative determination of soluble vegetal proteins through the Bradford method

After the developing period of 7 days, plantlets were cut in 1 cm pieces, separating the root from the plantlet (stem), in view of separately quantitatively analyzing the soluble vegetal proteins from the two parts of the plant. They were suspended in 10 ml phosphate buffer and sonicated for 30 seconds 3 times, with 5 minute breaks, the extract being then centrifuged and held at a 4 °C temperature for 4-5 days.

To quantitatively determine soluble vegetal proteins, same steps as in the case of extract from the dinitroderivates treatment were followed, pipetting the same reactive amounts, and reading after 3 minutes of incubation and 5 minutes of irradiation. Determining the total concentration of protein from enzymatic extracts was achieved with the Bradford method, treating extracts with Bradford reactive.

From Table 3 one can notice that in the case of the treated samples extracted from roots, the total soluble protein concentration has different values from the blank which does not contain metallic extracts. Therefore, specific activities vary based on the type of used metallic extract, the largest protein quantity being identified when treating with $HgCl_2$ and $NiCl_2$, while treating with $CuCl_2$, $Pb(NO_3)_2$ or $Cd(NO_3)_2$ determines enzymatic inhibition.

Table 3. *Specific activity values based on absorbance and protein concentration of the protein extract obtained from wheat plantlets roots*

	Weight of roots (g)	**Absorbance (595 nm)**	**Protein concentration, µg/mL**	**Specific activity**
Control	0.040	0.0798	0.159	0.0756
$CuCl_2$	0.050	0.0724	0.159	0.0756
$AgNO_3$	0.050	0.0747	0.159	0.0717
$HgCl_2$	0.050	0.0688	0.159	0.0663
$Pb(NO_3)_2$	0.050	0.0878	0.159	0.0756
$NiCl_2$	0.050	0.0680	0.159	0.0705
$Cd(NO_3)_2$	0.050	0.0695	0.159	0.0745

In the case of wheat plantlets extract, specific activity values show significant differences, compared to their evolution in the root extract (Fig. 2). This increasing variation of activities corresponding to the treatments with 7 types of toxic indicates that there is a much larger protein specific activity in wheat plantlets than in roots, varying according to the applied treatment. Consequently, a lower activity was identified in case of treating with $AgNO_3$ and $CuCl_2$ and a larger one was identified when treating with $Cd(NO_3)_2$, $HgCl_2$, $NiCl_2$ and $Pb(NO_3)_2$.

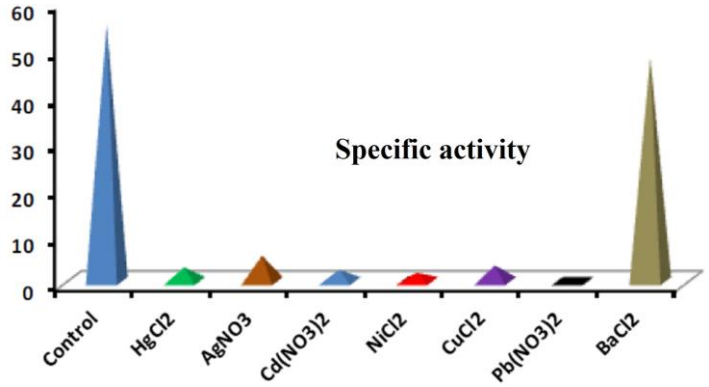

Fig. 2. *Specific activities of the protein extract obtained from wheat plantlets after treating with heavy metals*

Determining the peroxidase activity

The much more intense activity of peroxidase in the experimental batch can be clearly attributed to $NiCl_2$. In other words, Ni^{2+} interferes in the germination and growth processes of wheat plantlets. It is well known that the seed deterioration process starts immediately after harvesting, various intrinsic and extrinsic factors contributing to this. For this reason, to prevent rapid loss of vigor, viability and productivity, a controlled approach of their depositing is necessary, especially in environments where the deterioration rate is imposed by a hostile condition (high heat and increased humidity). Applying various treatments substantially slows down the deterioration rate and rapidly increases the seeds viability rate.

Tabel 4. *Peroxidase activity values in wheat plantlets after 7 days of treatment*

Treatment	UP/mg protein
Control	4.52
$HgCl_2$	7.52
$AgNO_3$	8.63
$Cd(NO_3)_2$	8.99
$NiCl_2$	15.98
$CuCl_2$	7.32
$Pb(NO_3)_2$	6.12
$BaCl_2$	5.73

Determining catalase activity

The intensification of the two enzymes (peroxidase and catalase) activity denotes the fact that the heavy metals treatment is capable of inducing a significant decoupling of the transport chain of electrons and of the oxidative phosphorylation, resulting in dissipating the membrane gradient and, consequently, increasing the production of oxygen reactive species. This explains the catalase activity intensification in plantlets treated with $NiCl_2$ (40.72 UC/mg protein), compared to plantlets from the control batch (15.18 UC/mg protein) (Fig. 3).

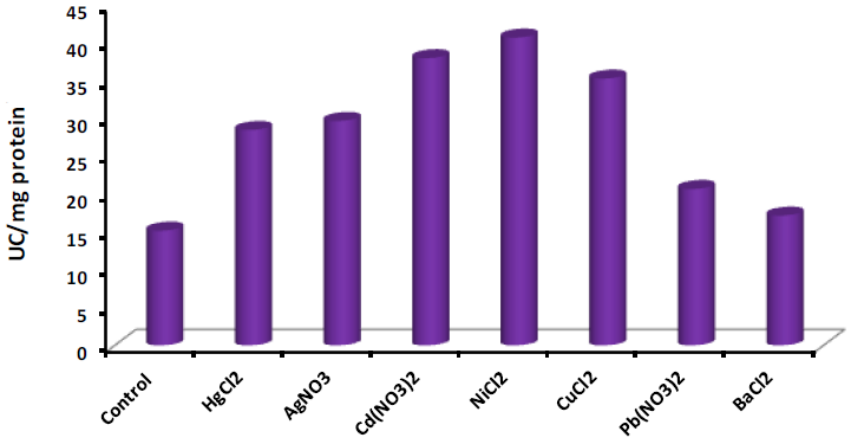

Fig. 3. *Determining catalytic activity from wheat plantlets exposed to 7 days of treatment*

4. Conclusions

The present paper proved the toxic effects of heavy metals on the viability of plants. This experiment revealed the sensitivity of wheat seeds to heavy metals, which inhibit the plants growth. Individual heights and the unitary average mass reflect the effects of various treatments more precisely. This could be explained by the fact that the effect owed to the number of unsprouted seeds is removed, only taking into account individual plants that had effectively suffered from the treatment. Also, these differences are also caused by the fact that each compound specifically influences each plant. Every living organism reacts specifically to exterior factors. Thus, dead seeds were not taken into account in case of individual height and unitary average mass.

Enzymatic activity of vegetal species was determined. Wheat extracts were concentrated and exposed to subsequent stages of concentration (ammonium precipitate) and dialyze. Enzymatic extracts stability was relatively high for short periods (1 week), but was substantially modified in case of storing for longer periods (remained enzymatic activity was 30-40% after 3 weeks).

Enzymes involved in respiratory processes are very attractive for understanding the evolution of life, from the prokaryote to the eukaryote stage, as well as for classifying pathological modifications that take place in malign processes, where the reversed

phenomenon of transition takes place (from normal respiration of cells to lactic fermentation, with all biochemical implications: pH modification of body fluids, degradation of ramified essential aminoacids etc.).

The method of enzymatic determinations can be successfully applied particularly in the case of plantlet roots, due to the more accentuated inhibition. The extraction degree is influenced by the dimensions of the plant parts. Enzymatic activity from various plant compartments significantly differs based on treatment or source. To conclude, the value of enzymatic activity in roots is superior to that in leaves, and the type of phenolic extract used influences the enzymatic activity.

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