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EMPHASIZING THE TOXICITY OF HEAVY METAL IONS ON PLANTS: MONITORING WITH AN AMINO ACIDS ASSAY

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ABSTRACT

In order to emphasize modifications on living organisms resulted from interaction with heavy metals, we applied a simple, sensitive, quick, inexpensive and reproducible method. It evaluates modifications through determining released amino acids. This method comprises three stages: treating plants with the investigated heavy metals, extracting resulted amino acids and dosing them with a ninhydrin reagent. The colored solution resulted from ninhydrin dosing is spectrophotometrically measured at 516nm. The Lambert-Beer law is valid within the concentration field of 0-20 μg mL^{-1}.

KEYWORDS: biostructure; determining amino acids; hypoxia; germination tests; heavy metals; ninhydrin reagent

1. Introduction

One of the main worldwide issues today is represented by the need of protecting the environment and human health. This requires prevention and monitoring the pollutant contamination of our environment and alimentary products [1].

Life, as presence and manifestation in the universe, is a paradox. On one hand it is a truism, manifesting through an amazing variety of forms and properties; on the other hand, the phenomena complexity overwhelms us in such a way that all proposed patterns are, *grosso modo*, invariably limitative and reductionist [2]. Also, life is characterized by a series of conservation functions, such as defense, motion and adapting, and species perpetuation functions, such as multiplication or reproduction. It is known that most animal creatures can't live more than several minutes without air to breathe, more than several days without water to quench thirst and maintain hydration, or more than several weeks without food to quench hunger. In absence of these supplies, life is not possible [3, 4].

Numerous physicochemical theories have been advanced to explain the nature of living matter, like the membrane, absorption and the association-induction theory. Modern biology has developed in light of molecular conception, but has not succeeded in offering a satisfactory explanation regarding the nature of living matter yet. It does not differentiate between 'dead' and 'alive'. Moreover, these theories are not detailed enough for all obtained experimental data [5].

Metals with toxic potential usually present multiple harmful effects, affecting most tissues and organs and interfering with the enzymatic processes that ensure a proper organism functioning. Human exposure to metals is produced during their passing through the physical and biologic environmental compartments; the physical and chemical form of metals can change, the concentration ratio can be altered, so that metals can selectively accumulate in the trophic chain [6, 7]. Man is always exposed to a complex mixture of elements that are found in aliments, the main metal exposure source. Toxicity and metabolizing are strongly dependent on diet factors, like the chemical composition of aliments and the protein source nature. In order to estimate the potential of metallic interactions in living organisms, it is necessary to examine the relations between the ionic properties and the ions capacity of binding to organic ligands. The type of amino acids from the area of affinity with metallic ions of metalloenzymes defines, at least partially, the category of metallic ion that will be bound and will correspond to their function [8, 9].

Stress provoked by heavy metals is a major problem which affects agricultural productivity. Natural flora presents differences of tolerance for heavy metals. Some plants develop well in a soil

enriched with toxic heavy metals, while others can't develop at all [10, 13].

In consequence, we used a simple method to monitor the state of living organisms, state which is dependent on their biostructure integrity. This paper refers to a spectrophotometric amino acids determination assay. Amino acids are extracted in 1M sucrose solution form plant's biostructure, in order to emphasize a normal state or one altered by heavy metals [14].

2. Materials and methods

Reagents used for research were of analytical purity, while water solutions and suspensions were prepared with bidistilled water of high purity (milliQ, R = 18.2 Ω).

Treatment solutions: $HgCl_2$, $NiCl_2$, $Cd(NO_3)_2$, $Pb(NO_3)_2$, $BaCl_2$, $CuCl_2$ and $AgNO_3$ with concentration $2x10^{-3}$M. Blank was distilled water.

Ninhydrin reagent. 0, 4 g of ninhydrin and 0, 4 g of $Cd(NO_3)_2 \cdot 4H_2O$ were dissolved in 25 ml buffer solution with pH = 5, 5. Completion up to 100 mL was done with glycerin. Solution is prepared extemporaneous.

Buffer solution. 54, 4 g of crystalized sodium acetate was dissolved in approx. 50 mL of heated distilled water. After cooling, 10 mL of glacial acetic acid was added, as well as distilled water up to 100 mL. If necessary, pH value is corrected at 5, 5 with glacial acetic acid, respectively with sodium hydroxide.

Alcoholic solution, 80 % (v/v).

Amino acids standard solutions. A stock solution with concentration of 1 mg/mL was prepared by dissolving 0, 1 g from every amino acid in 100 mL of distilled water. Standard solutions were obtained through their corresponding dilutions. Calibration curves were traced with alanine.

Other amino acids solutions. In order to verify the proposed method, solutions containing 20µg mL^{-1} mixture of different amino acids were taken into account.

Biological material. Corn seeds (*Triticum aestivum*), Gasprom sort, 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.

Germination determining. Lots of 20 seeds each were treated with different solutions of biostructural inhibitors, 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.

Seeds are watered daily with 5 mL of bidistilled water. After 7 days, germinated, abnormal and dead seeds are counted, as well as resulted plantlets. Plants are cut from the seed level, then measured and weighed (height, H, in cm and mass, m, in g).

Extracting released amino acids. Corn plantlets (3-5 g) were harvested from the seed level and had their base soaked in 10 mL of distilled water (blank) or in the treatment solution for 1 hour. Also, during the experiments, the leaf samples were cut in 1 cm-long pieces and soaked in treatment solution for 1 hour. Next, treated samples were dabbed with filter paper and carefully introduced in appropriate tubes. Extraction solutions were added (10 mL or 20 mL) in the tubes that had been obliquely held so that the biological material remained soaked in liquid. Tubes were slowly rotated from time to time during the entire extraction. The method can be used to prove the effect of hypoxia on plants biostructure for the first time.

Determinations. 1 mL of amino acid extract from seeds or plants was pipetted in regular tubes. 1 mL of ninhydrin reagent was added.

The mixture was energetically stirred. Next, tubes were maintained in a 100 °C water bath for 60 minutes, then cooled at room temperature. 5 mL of alcoholic solution were added in each tube.

After stirring, the colored solution absorbance was read at 516 nm in quartz cuvettes against the blank. In the same conditions, the blank solution remained colorless.

Calibration curve was traced in the 0 – 20 µg mL^{-1} domain with alanine.

Statistic calculus. The standard deviation (S) and the standard deviation of the mean (sx) were calculated.

3. Results and discussions

Testing structural modifications undergone by corn seeds exposed to heavy metals treatment can be done easily, economically, quickly and spectacularly by using corn seeds exposed to germination. Germination determining will follow these steps: lots of 20 seeds each will be treated with different solutions of biostrutural inhibitors, then left to germinate on filter paper in Petri dishes. Treatment duration is 1 hour, after which seeds are uniformly disposed in Petri dishes, on double filter paper, together with the treatment solution.

Seeds are to be watered daily with 5 mL of bidistilled water.

After 7 days, germinated, abnormal and dead seeds are counted, as well as the resulted plantlets. Then, plantlets are cut from the seed level, measured and weighed (height, H, in cm and mass, m, in g).

Germination tests with various heavy metals of concentration 2x10^{-3} M.

Solutions of $Pb(NO_3)_2$ and $BaCl_2$ of concentration 2x10^{-3} M inhibit corn growing, whereas compounds with Hg^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} of the same concentration have proved to be toxic for corn seeds (Fig. 1). In principle, watery solutions of Pb^{2+} and Ba^{2+} have approximately the same effect on corn seeds as the blank treatment (distilled water). Using the others metals leads to a decrease in plantlets height. Toxicity can be produced by the effect of Hg^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} on seed developing, which can be more toxic than solutions of $Pb(NO_3)_2$ and $BaCl_2$.

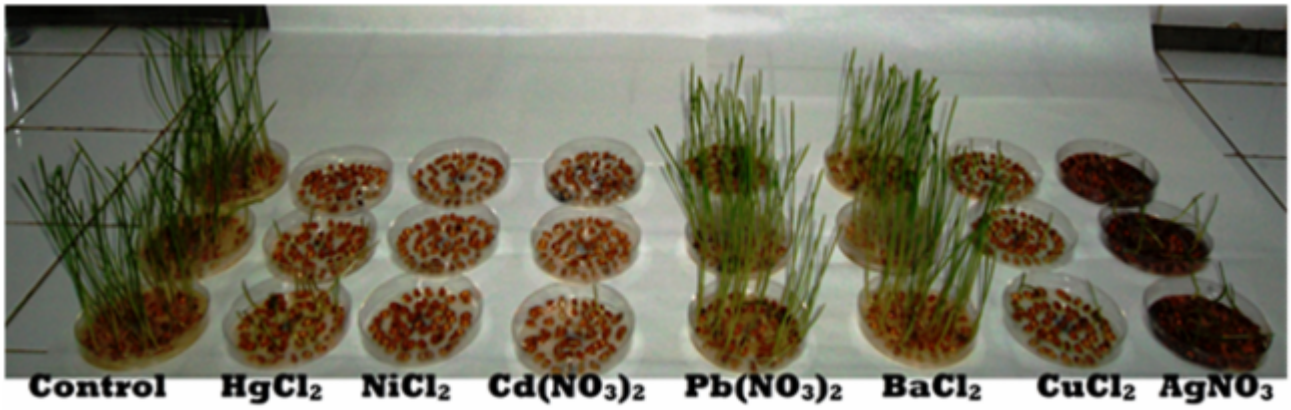

Fig. 1. *Effect of heavy metals on corn seeds during the 7 day - germination process*

Table 1. *Effect of heavy metals on corn seeds germination (50 seeds)*

Treatment *)	Plantlets	No. germinated seeds	No. dead seeds	Length plantlets (cm)	Weight plantlets (g)
Control	35.66±0.33	0.66±0.3	13.66±0.33	2.40±0.09	393.10±11.26
$HgCl_2$	11.66±0.66	3.33±0.33	35±7.57	0.33±0.21	30±1.3
$NiCl_2$	0	1.66±0.33	48.33±0.33	0	0
$Cd(NO_3)_2$	1±0.33	11.66±2.84	37.33±3.52	0.01±0.001	1.94±0.15
$Pb(NO_3)_2$	28.66±3.38	1.33±0.01	20±2.08	1.87±0.56	272.43±6.22
$BaCl_2x2H_2O$	35.43±0.88	0.66±0.01	12.36±0.88	2.315±0.2	360.13±10.09
$CuCl_2$	4.66±0.33	3.33±1.33	42±1.52	0.09±0.01	11.83±1.1
$AgNO_3$	9.33±3.17	6±1.73	34.66±3.75	0.28±0.1	37.60±5.98
*) mean of three independent values					

In this experiment, it is clearly noticeable that heavy metals have an inhibitory effect upon corn plantlets height (Table 1). For example, the most toxic heavy metals have proved to be Ni (H = 0 cm) and Cd (H = 0.013 cm). However, Ba had approximately the same stimulating effect as distilled water (length = 2.315 cm, and respectively 2.40 cm).

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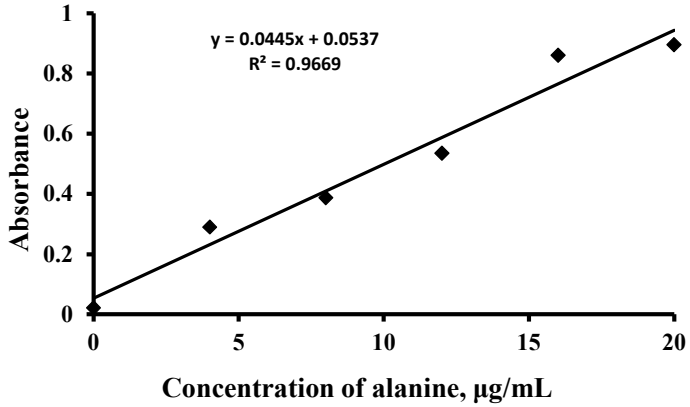

Fig. 2. *Calibration curve when determining amino acids from the extraction solution*

Calibration Curve. Absorbance of colored solutions was proportional with the alanine concentration within the entire concentration field of 0–20 μg·mL^{-1}, its increase being linear (Fig. 2). When others amino acids were used, different calibration curves were obtained.

The maximum absorbance was measured at glycine, and the minimum at hydroxyproline (Table 2). Therefore, because analyzed samples have different amino acid compositions, the calibration curve must be traced with the most suitable amino acid for each sample.

The molar absorption coefficient of the amino acid and of the amino acids mixture from the extraction solution should have similar values.
Thus, the exact amino acid which is used for achieving the calibration curve needs to be mentioned.

Table 2. *Absorbance and molar absorptivity of certain amino acids in conditions of ninhydrin reaction*[a]

Amino acid	Absorbance	Molar absorptivity	Absorbance (%, 21 hours later)
Alanine	0.790 ± 0.035	$1.758 \cdot 10^3 \pm 77.9$	–7.3
Lysine	0.447 ± 0.018	$1.631 \cdot 10^3 \pm 67.3$	–5.3
Tryptophan	0.332 ± 0.016	$1.696 \cdot 10^3 \pm 80.1$	–5.0
Phenylalanine	0.415 ± 0.014	$1.717 \cdot 10^3 \pm 55.8$	–10.0
Arginine	0.372 ± 0.018	$1.619 \cdot 10^3 \pm 79.4$	–3.0
Isoleucine	0.480 ± 0.017	$1.571 \cdot 10^3 \pm 56.8$	–2.0
Norleucine	0.542 ± 0.020	$1.775 \cdot 10^3 \pm 65.6$	–6.7
Leucine	0.551 ± 0.023	$1.806 \cdot 10^3 \pm 75.7$	–4.3
Glycine	1.150 ± 0.045	$2.156 \cdot 10^3 \pm 85.8$	–12.0
α-Aminobutyric acid	0.628 ± 0.030	$1.616 \cdot 10^3 \pm 78.8$	+2.8
γ-Aminobutyric acid	0.449 ± 0.025	$1.156 \cdot 10^3 \pm 65.5$	–7.4
Serine	0.862 ± 0.035	$2.263 \cdot 10^3 \pm 89.8$	–5.0
Cystine	0.430 ± 0.015	$2.580 \cdot 10^3 \pm 90.7$	+3.7
Histidine	0.573 ± 0.023	$2.224 \cdot 10^3 \pm 88.7$	–2.1
Methionine	0.257 ± 0.018	$0.957 \cdot 10^3 \pm 67.5$	+6.0
Cysteic acid	0.435 ± 0.016	$2.032 \cdot 10^3 \pm 73.2$	–2.0
Proline	0.235 ± 0.017	$0.758 \cdot 10^3 \pm 55.8$	+2.8
4-Hydroxyproline	0.055 ± 0.014	$0.181 \cdot 10^3 \pm 44.5$	+3.7
Asparagine	0.505 ± 0.022	$1.662 \cdot 10^3 \pm 71.4$	–10.7
Valine	0.501 ± 0.023	$1.660 \cdot 10^3 \pm 72.3$	–2.0
Mean	0.500 ± 0.022	$1.640 \cdot 10^3 \pm 72.1$	–3.3

[a] Mean of four replicate analyses; 20 μg mL^{-1} amino acid.

Through this method, a solution of an amino acid mixture with concentration of 20 μg mL^{-1} was analyzed, similar to that from corn plantlets (Table 3). The molar absorption coefficient for the corn amino acids mixture was 1.747 x 10^3 (mean molecular mass being 117.4 and the molar absorption coefficients being taken from Table 2). Hence, the most suitable amino acid for calibration in case of this amino acid mixture would be alanine (absorbance = 0.790; molar absorption coefficient = $1.758 \cdot 10^3$ L mol^{-1}cm^{-1}), because the molar absorption coefficient of this amino acid and of the mixture from the extraction solution have proximate values (Table 4).
Interferences.

Effect of coexistent species was studied using an alanine solution of concentration 20 μg mL^{-1}. The method proved to be weakly affected by other ninhydrin-positive substances. Still, ammonia, ammonium ion, amines etc., called ninhydrin-positive substances, also react with ninhydrin. Since plants biostructure is investigated with this method, these ninhydrin-positive substances do not dramatically affect results considering their low concentrations.

Reproducibility and sensitivity. The method is reproducible, and the Lambert-Beer law is respected within the concentration interval of 0-20 μg mL^{-1}.

Effect of various reaction conditions. Effect of reagents and reaction time and temperature upon coloring intensity were studied after the amino acid extraction in the 1M sucrose solution. In view of a complete color reaction, using 1 mL of ninhydrin reagent solution was sufficient.

The resulting colored compound has an absorbance maximum at 516 nm (500-530 nm). A minimum of 60 minutes was necessary for the color to maximally develop at 100 °C.

Table 3. *Absorbance of an amino acids mixture similar to that of corn plantlets*

Amino acid	Concentration (μg mL^{-1})	Relative absorbance
Cysteine	0.63	0.027
Asparagine	7.80	0.201
Histidine	1.35	0.039
Aspartic acid	0.63	0.066
Glycine	1.70	0.043
Serine	0.21	0.060
Glutamic acid	0.25	0.022
Threonine	0.47	0.013
Alanine	3.03	0.118
Tyrosine	0.73	0.015
Methionine	0.75	0.033
Phenilalanine	2.70	0.056
Leucine	0.75	0.120
Mean	40.00	0.813

Table 4. *Values obtained from amino acid extraction from plantlets treated with Pb^{2+} and Ba^{2+} (λ=516 nm)*

	Control			$Pb(NO_3)_2$			$BaCl_2 \cdot 2H_2O$		
Absorbance	0.773	0.901	0.879	0.901	0.858	0.887	0.397	0.363	0.795
Absorbance	0.887	0.669	0.852	0.942	0.516	1.038	1.429	0.66	0.465
Absorbance	0.780	0.724	0.888	1.009	0.961	0.930	1.277	1.221	0.643
Mean	**0.813**	**0.764**	**0.873**	**0.950**	**0.778**	**0.951**	**1.034**	**0.748**	**0.634**
Errors	**0.07**	**0.06**	**0.01**	**0.03**	**0.13**	**0.04**	**0.32**	**0.25**	**0.09**

The colored solution's absorbance increased 7.5 times at a reaction time rise from 5 to 60 minutes (Fig. 3). Absorbance then halved when the reaction time was prolonged to 120 minutes, indicating decomposition of the formed colorant.

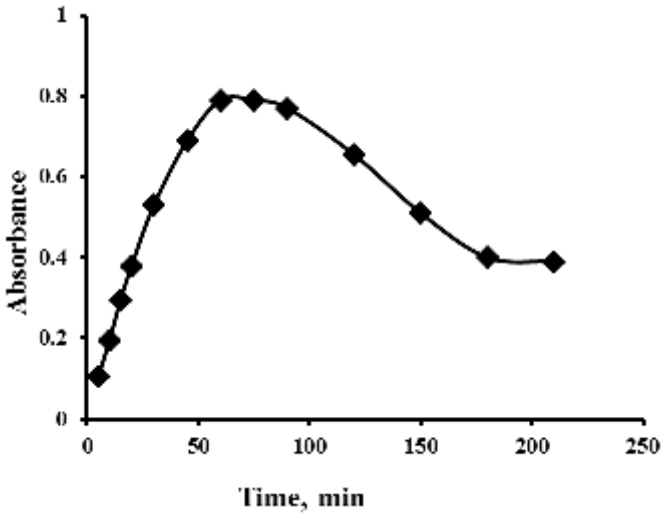

Fig. 3. *Effect of time upon ninhydrin-amino acid coloration intensity (100°C, 20 μg mL^{-1} alanine)*

4. Conclusions

The chosen method is applicable when monitoring the degradation of plants biostructure through determining amino acids extracted from 1M sucrose solution. The method can also be useful for environmental analyzes (pollutants), as well as within biochemistry laboratories in general. It requires simple and easily acquirable equipment, inexpensive chemicals and glassware, and personnel with average qualifications.

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