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BIOCHEMICAL AND MOLECULAR APPROACHES IN *Raphanus satives* AFTER HEAVY METALS (COPPER & CADMIUM) EXPOSURE

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ABSTRACT

Raphanus sativus **was chosen for studying the toxic effect for two elements (cadmium & copper) on some biochemical & molecular characteristic, so cadmium concentration (0, 5, 10, 15) ppm were determined from Cd $(NO_3)_2$ in addition to control (D.W.) as triplicate for each concentration and *Rafanus* seed irrigate after implementation in fertilized soil for 60 days.**

On the other hand, Copper concentration was (0, 10, 15, 25) ppm which previously prepared from $(Cu(NO_3)_2)$ in addition to control group represented by D.W.

Regarding soil, pH, E.C., Nitrate, soil texture values were determined to detect the soil characteristics before and after agriculture.

The results have confirmed at the end of the experiment and from plant species analysis that copper concentration and cadmium concentration in irrigation water were lead to change in ROS, SOD, CAT, MDA, GSH-px, total protein, total sugar, proline, chlorophyll & moisture content in Raphanus.

Two elements concentration (Cu, Cd) were measured in shoot & Root system studied prepared concentrations with control group so the copper concentration in control group after Cu exposure was higher than other group and also the concentration in root system was the highest in comparison to shoot system.

Molecular Results showed these concentrations, which have no effect on DNA status i.e. that no damage to DNA in plant with concentration increasing and this compatible with no significance effect on *als* gene amplification and more amplified was in 15 mg/l for Cu & 10 mg/l for Cd.

Keywords: *Raphanus satives*; heavy metals; biochemical and molecular characteristic.

INTRODUCTION

Soil contamination with heavy metals has become a world-wide problem, leading to the loss in agricultural productivity. Plants have a remarkable ability to take up and accumulate heavy metals from their external environment and it is well known that high levels of heavy metals affect different physiological and metabolic processes [1]. On the other hand, heavy metals stored in plants may have toxic effect on animal and human organisms when taken through the food chain.

Heavy metals are considered to be an important stress factor for plants.

If a natural amount of heavy metals is present in soils, plants are able to avoid their negative impact [2]. Plants have high potential to accumulate heavy metals, which is useful in bioremediation [3,4,5].

However, high concentrations of heavy metals cause harmful effect on cellular and physiological processes in plants [6,7].

Plants have a remarkable ability to take up and accumulate heavy metals from their external environment and it is well known that some of these metals such as Cu, Zn, Mn, and Fe at trace levels are required for normal plant growth and development [8], since they are structural and catalytic components for proteins and enzymes production.

The impact of heavy metals is strongly related to their doses, the plant species and the plant developmental phase as well as environmental factors characteristic for a given climate zone [9].

In various plant species subjected to heavy metal stress there is a variety of studies that reported the antioxidant activity effects on plants [10,11].

Among pollutants of agricultural soils, Cu has become increasingly hazardous due to its involvement in fungicides, fertilizers and pesticides. Cd is not an essential nutrient and it is one of the heavy metals that are known to generate toxicity even at a very low concentration, because accumulation in plants, during growth, take place in edible parts, thereby, endangering yield and quality crop [12].

MATERIALS AND METHODS

The Study Samples

Soil preparation for agriculture

Soil samples were collected at an area of agricultural soil whose fertility is known and then distributed in plastic pots, placing 1 kg of soil in each. Seeds of *Raphanus sativus* were used as study plant. The total of pots were divided into two sets as follow:

A- Watered with solution of Cu $(NO_3)_2$ in 0, 10, 15, 25 ppm concentrations. With three replicates per concentration plus 3 replicates for control.
B- Watered with solution Cd $(NO_3)_2$ in 0, 5, 10, 15 ppm concentrations. With three replicates per concentration plus 3 replicates for control.

These A and B assays were conducted during 60 days.

Environmental Parameters

pH

Hydrogen Ions were measured by a pH meter Type Hanna (Portugal) after calibration using standard solutions provided with the instrument In the soil extract 1:1 [13].

Electrical conductivity (E.C., µs/cm)

A conductivity meter in Spilled saturated soil paste was used to measure the Electrical Conductivity in spilled saturated soil paste.

Soil texture (Gm / L)

It was estimated using Hydrometer capacitor (ASTM) (15H) following the method described in [14].

Soil nitrate (NO_3) concentration (ppm)

This anion was determined by chromatic acid after nitrate extraction with copper sulphate (0.02) following the procedure reported by [13].

Table 1. Primer pairs, sequences and amplicon size

Plant species	Primers		5'-sequence-3'	Amplicon size (bp)	Reference
Raphanus sativus	*als*	F	'5-GCA GAT GCT TAT GCA CG -3'	382 bp	(M.K.Tan and R.W.Medd 2002)
		R	'5-CTC GTC GAG GAC CTG AAT CG -3'		

Biochemical Parameters for Plant

The amount of ***chlorophyll*** was estimated in plant leaves using a chlorophyll measuring device, a chlorometer and values reported in SPAD units.

The concentration of ***proline*** was determined by the method reported in [15].

Total sugar concentrations were estimated by the extraction method finination [16].

The protein content was estimated by the [17] method using dry leaves.

Preparation of Plant Extracts for Antioxidant Enzymes Determination

A method described by [18] was used to prepare the plant extract. It was taken 0.1 g of fresh leaves, crushed in a pre-cooled ceramic veneer placed on ice packs to prevent enzyme breakdown, with addition of 1 ml of the phosphate buffer solution and added 0.03 g of Polyvinyl pyrolliodone (PVP). Centrifugation at 10,000 cycles 10 minutes under 4°C.

Reactive Oxygen Species (ROS) are determined using the method reported by [19] and expressed in terms of micro molar hydrogen peroxide equivalent per liter (µmol H_2O_2 Equiv./L).

Plant enzymes measure**:** Four types of enzymes were determined: Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GHS-px) and Malondialdehycde (MDA); by using enzyme linked immunosorbent assay (sandwash elisa) technology according to manufacturing company (PARS BIOCHEM, CHINA).

Heavy Metals Determination

Digestion of plant samples (root and shoot) was done using a method reported by [20] It was weighted 0.2 g of dry plant samples, placed in a digestion flask, adding 3 ml of sulfuric acid; after 1 day it was added 1 ml of acid mixture (sulfuric acid + perchloric acid), the flasks are placed on digestion and the fumes are then detected, and the color of the samples gradually changes until a colorless solution is obtained; then cool the samples and add distilled water (DDW?) up to obtain a 50 ml sample.

Molecular Parameters Determination

This assay has been done using the OxiSelect™ Comet Assay Kit (3-Well Slides) Cat. No. STA-350) with the purpose of identify DNA damage.

Gene Emplification

DNA extraction Kit (Favorgen) used to identify DNA fragment and after extraction, DNA samples visualized by Electrophoresis.

Polymerase chain reaction (PCR) of Gene *als*.

RESULTS

Soil Texture

According to the percentages for soil content A% sand, B% clay, C% lime and using a triangle texture, the soil classifies as Medium loam.

Table 2. Environmental parameters of soil in *Raphanus satives*

Parameters	Concentration of Cu (ppm)			C	Concentration of Cd (ppm)		
	10	15	25		5	10	15
pH	7.68	7.63	7.52	7.95	7.75	7.78	7.66
E.C (µs/cm)	4.44	5.77	7.96	4.44	5.60	5.11	5.07
Nitrate (ppm)	144.6	138.11	140.94	121.6	142.25	142.159	127.28

If the C represents the control reference therefore you should refer how the soil was affected by adding the nitrate solutions. Also, do you register the pH and conductivity

Measurement of Heavy Metals Concentrations in Shoot and Root:

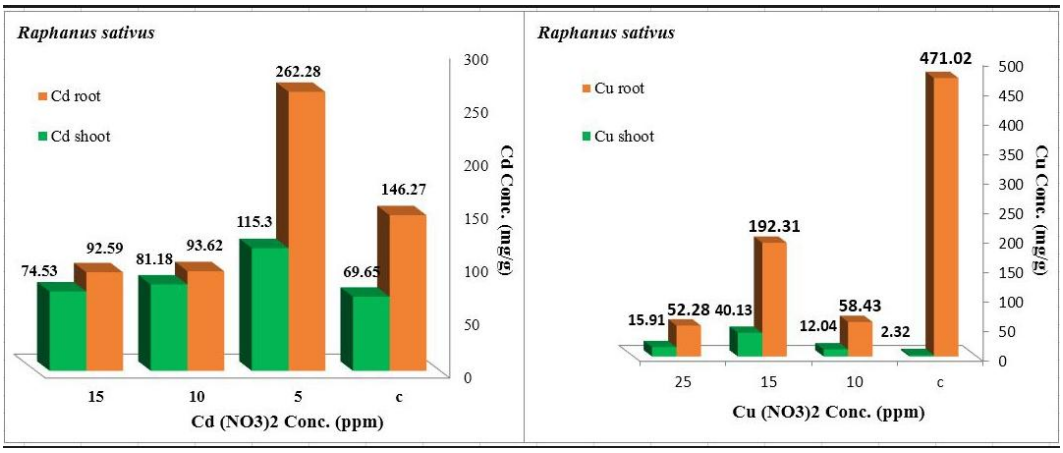

Fig. 1. Concentration of heavy metals for shoot and root of *R. sativus* after exposure of copper and cadmium

Chart 1. Biochemical parameters

Test	Elem.	conc	M.± Std. Deviation	Test	Elem.	conc	M.± Std. Deviation	Test	Elem	Con.	M.± Std. Deviation	Test	Elem	Con.	M.± Std. Deviation
ROS	Cu	0	0.5690±0.11001	GSHpx	Cu	0	3.7923±0.16811	MDA	Cu	0	0.2497±0.03758	PROLINE	Cu	0	4.6789±0.85640
		10	0.8845±0.07750			10	3.3600±1.43700			10	0.8645±0.07750			10	3.0730±0.21826
		15	0.5120±0.00100			15	4.7040±0.00100			15	0.2550±0.01700			15	3.6895±0.07101
		25	0.5660± 03900			25	1.5520±0.00100			25	0.1783±0.00503			25	4.1240±0.04709
	Cd	0	0.5690±0.11001		Cd	0	3.7923±0.16811		Cd	0	0.2497±0.03758		Cd	0	4.6789±0.85640
		5	0.5590±0.00100			5	3.6610±0.00100			5	0.1200±0.01000			5	6.2156±0.38458
		10	0.5270±0.00100			10	4.1940±0.30100			10	0.1710±0.00100			10	6.6860±0.42108
		15	0.3880±0.00100			15	6.9290±1.02000			15	0.1325±0.02350			15	6.6199±0.25751
LSD(0.05)		S	0.089792	LSD(0.05)		S	0.908996	LSD(0.05)		S	0.049892	LSD(0.05)		S	0.69717
SOD	Cu	0	13.3600±2.71369	CHLORO	Cu	0	43.7167±1.89363	SUGARS	Cu	0	119.3600±34.35361				
		10	0.8845±0.07750			10	47.6333±2.25019			10	116.7000±51.73654				
		15	7.3550±0.34300			15	42.0333±2.25019			15	102.3333±33.06488				
		25	7.9980±3.50200			25	36.3333±5.17333			25	81.1233±53.19037				
	Cd	0	13.3600±2.71369		Cd	0	43.7167±1.89363		Cd	0	119.3600±34.35361				
		5	5.7160±0.07600			5	31.4000±4.33474			5	121.3867±25.25938				
		10	4.3440±0.00100			10	38.0167±6.08612			10	157.4350±7.31500				
		15	3.7340±0.00100			15	35.9667±7.22311			15	111.3725±50.00250				
LSD(0.05)		S	2.624893	LSD(0.05)		S	7.480101	LSD(0.05)		NS					
CAT	Cu	0	1.2600±0.06707	FRESH	Cu	0	88.1667±1.15036	PROTEIN	Cu	0	27.1800±14.80653				
		10	0.8845±0.07750			10	87.3333±2.48261			10	22.8233±9.17073				
		15	1.6250±0.03500			15	89.2000±0.60828			15	27.4467±19.03719				
		25	1.4835±0.19450			25	88.7333±0.80208			25	15.1367±1.44001				
	Cd	0	1.2600±0.06707		Cd	0	88.1667±1.15036		Cd	0	27.1800±14.80653				
		5	1.5455±0.30950			5	88.3333±1.36137			5	11.2667±1.27970				
		10	1.7310±0.07100			10	89.3333±1.92180			10	17.4467±3.94544				
		15	1.1655±0.14150			15	89.4667±0.81445			15	48.2933±0.61101				
LSD(0.05)		S	0.214479	LSD(0.05)		NS		LSD(0.05)		S	15.15968				

Raphanus sativus

Comet assay:

Table 3. Comet parameter of DNA damage in *Raphanus sativus* indicating of extent of DNA fragmentation induced by cadmium and copper

Conc. / element	control			10 ppm			15 ppm			25 ppm		
Cu	Damage 0.2	COMET L	40.7±7.6	Damage 0.105	COMETL.	41.7±5.37	Damage 0.64	COMET L	49.32±8.31	Damage 0.17	COMETL.	72.34±14.69
		TAIL L.	3.6±1.83		TAIL L.	5.98±2.16		TAIL L.	7.24±3.57		TAIL L.	13.6±6.30
		Tail M.	1.26± 0.17		Tail M.	0.629±0.32		Tail M.	0.47±0.54		Tail M.	2.42±1.29
Cd	Control			5 ppm			10 ppm			15 ppm		
	Damage 0.2	COMET L	40.7±7.6	Damage 0.37	COMETL.	38±7.8	Damage 0.40	COMET L	27.3±7.6	Damage 0.46	COMETL	30.3±4.69
		TAIL L.	3.6±1.83		TAIL L.	4.9±3.54		TAIL L.	5.5±3.86		TAIL L.	6.7±3.7
		Tail M.	1.26± 0.17		Tail M.	0.825±0.59		Tail M.	1.52±1.33		Tail M.	2.77±3.2

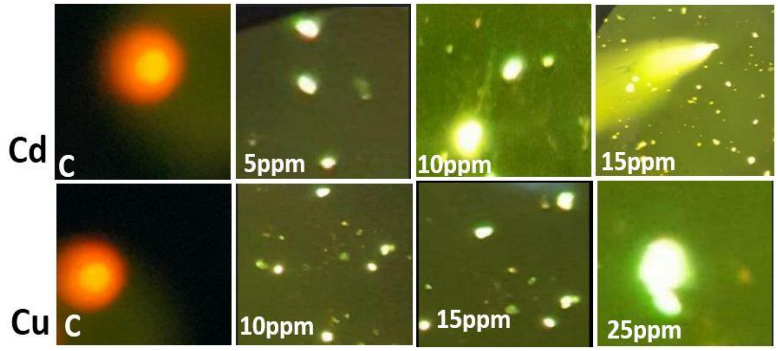

Fig. 2. Representative images of damaged nuclei (40X) in *Raphanus sativus* induced by cadmium and copper

PCR Product to Amplification of *als* gene:

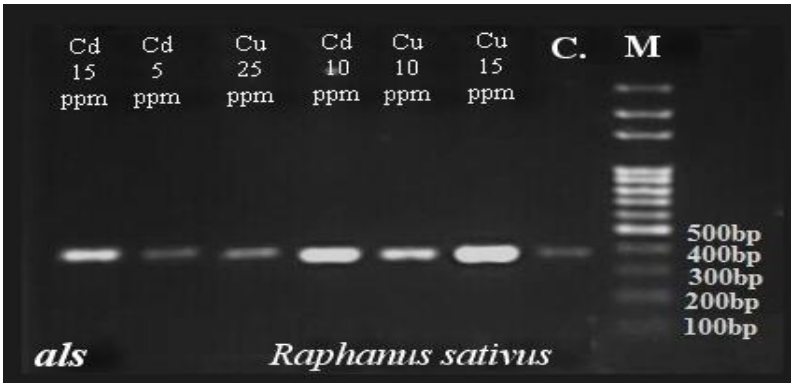

Fig. 3. 1.5% agarose gel electrophoresis at 72 volt for 60 minutes of PCR to *als* amplicon (382 pb); lane M represent DNA marker size (100 pb)

DISCUSSION

The results for the studied plant *Raphanus satives* were pH neutral and ranged between (7.4 -7.9) in, the values of electrical conductivity ranged betwee(4.4–7.9) (µs/cm) and Nitrate values were convergent in *Raphanus satives* where nitrate values in control unit 121.6 were increased by nitrate values by varying concentrations of copper and cadmium. The agricultural soil used in the experiment is characterized by a slight salinity and basal pH, which is characteristic of agricultural areas in central Iraq [21].

The soil was fertile because of its nitrate content, as the soil content of nitrates was more than (121.6). These values affected the plant content after exposure to the elements (cadmium and copper). The protein and proline, which is the nitrogen a key component of their formation and was derived from the soil [22].

The results for concentration of heavy metals for shoot and root of plants after exposure of copper and cadmium showed higher absorption of cadmium in *Raphanus satives* although there is the varying values for all concentration in this plant compare with copper, Attributable the higher root absorption more than shoot in both plants and, as we can see, in Fig 1 is one of the defense methods of the plant against the stress of heavy elements [23].

The reason for the decrease in shoot concentrations in the treated plant tissue compared to the control group due to the phenomenon of antagonisms between the heavy element and alkaline medium [24].

Proline is the first indicator to increase when the plant is not stressed [25]. In many of the previous studies, the height of the plant exposure to any concentration of heavy elements and this is confirmed by the results of the current study and that the presence of differences in the concentration of proline after the exposure to two elements in *Raphanus* Evidence that the plant can withstand this stress with the help of amino acid proline [26].

The path of biocides in plant metabolism is the stabilization of carbon dioxide (Calvin cycle) and the metabolic transformation of monoclonal sugars into all parts of the plant where they are stored or used [27].

The results showed a clear increase in protein when *R. sativus* were exposed to the cadmium component and recorded the highest value with high concentration of cadmium the reason for the plant's tolerance to high concentration of heavy elements may be due to the preservation of proteins within the plant [28].

Of all the main enzymes involved in oxidative stress defense, like SOD, APX, CAT and peroxidases, published reports describe both an increase in its activity and a decrease (or no change), depending on plant species, plant organ, type of metal, duration of the treatment, plant age, and growing media [29]. The results were varied for *R. sativus* plant for antioxidant enzymes after exposure to heavy elements, to determine the effect of heavy metals on certain gene *als* for *Raphanus* Acetolaceate synthase (ALS) is the first common enzyme in biosynthesis of the branched chain amino acids –valine, leucine and isoleucine [30]. Results showed no damage to the gene in these concentrations, To demonstrate the effect of heavy metals, it caused damage to DNA by used Comet assay, The results showed that there was a merger in some cells but the percentage did not reach the effect of DNA in *Raphanus*.

CONCLUSIONS

FL actuations in biochemical and molecular response after heavy metals exposure, Heavy metals don't reveal any considerable effect on plant species according to their concentrations, Non response in some biochemical parameters returns to accumulation of heavy metal and chelating mechanism by plant species. Molecular markers confirmed that these concentrations of heavy metals don't have considerable effect.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any

litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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