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Effect of Artificially Supplied Chromium on Glutamine synthetase & Glutamate Synthase Activity in Leaves of *Helianthus annuus* L. Varieties

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ABSTRACT: Release of heavy metal pollutants in soil is major threat to environment and crops. Heavy Metal pollutant, hexavalent chromium released due to anthropogenic activities effects, growth metabolism and enzymes of nitrogen metabolism in oil seed crop Helianthus annuus, after entering its to plant system. Chromium (VI) ranging from 0, 10, 20, 50, 100 and 200 mg kg^{-1} soil mixture was provided to plants of three varieties of sun flower and activity of glutamine synthetase and glutamate synthase enzymes was observed in leaves of mature plants. It was observed that even at the highest concentration of chromium, the enzyme activity was reduced by only 17.65 % and 18.22 % showing that glutamine synthetase activity was showing that glutamine synthetase activity was insensitive to chromium in all these varieties of plants. For glutamate synthase enzyme, the low chromium concentration of 10 and 20 mg kg^{-1} soil was favourable but high chromium IV concentration of 50, 100 and 200 mg kg^{-1} soil was adversely affecting the glutamate synthase activity in all the three verities of sunflower.

Keywords: Phytoremediation; Chromium; Sunflower; Glutamine synthetase and Glutamate synthase.

INTRODUCTION: Agriculture land and open fields most of the times are exposed to air, water and soil pollutants released from automobile exhaust sewage water industrial effluents including pesticides and chemical fertilizers. (Pandey et al., 2007). Among the variety of pollutants, heavy metals are responsible for posing serious threat to plant growth production of economically important crop by reducing quality and quantity of agriculture yield (Bisheh Kolari et al., 2011). Metal like Chromium (Cr), Cadmium (Cd), Mercury (Hg) and Lead (Pb) when present in higher concentration may affect the plant growth and productivity (Najafian et al., 2012). Due to increased population, industrialization and urbanization, concentration of these pollutants also has increased many folds and among these pollutants Cr(VI) has become a major concern because of its greater toxicity and mobility as compared to Cr(III), (Guptas et al., 2009). Cr (VI) is produced during industrial process and is found in all phases of environment including air, water, rocks and soil (Pandey et al., 2008). Most of human activities like mining, thermal power stations, electroplating, wood preservation, iron and steel manufacturing, textile industry, leather tannaries, paint industries, combustion of coal and petroleum release toxic chromium in the environment (Gheju et al., 2009). Phosphate fertilizers and fly ash also add chromium to soil (Parveen et al., 2011).

Growth and metabolism in the plants is affected by so many essential elements. Among these essential elements nitrogen is very important key element for the plants, responsible for growth and metabolism (Gangwar & Singh 2011). Various toxic heavy metals present in the soil severely affect the enzyme for nitrogen metabolism resulting in reduced crop yield. Dixit et. al, (2002) reported that chromium is toxic to plants and is responsible for reduced seed germination, leaf chlorosis, reduced pigmentation, stunted growth, damage of root cell etc. Contamination of heavy metal Cr(VI) in agriculture soil is a big problem in India's agriculturally and industrially rich states like Haryana and Punjab. Studies on the use of metal hyper accumulator crop plant species for the phytoremediation to Cd(II) and use of EDTA for chemically assisted phytoextraction using Indian mustard (*Brassica juncea*) were carried out by Jiang et al.(2003). January et.al 2008, examine oil seed crop Sunflower (*Helianthus annuus* L.) as hyperaccumulator of Cd, Cr, Ni, As and Fe. Sunflower is amongst the world's top four oil seed crop grown in many parts of the

world. Not much work has been reported on the effect of Cr(VI) on enzyme of nitrogen metabolism in oil seed crop like sunflower. Present investigation deals with the effects of variable dozes of Cr(VI) on Glutamine synthetase and Glutamate synthase activity in leaves of mature plants of *Helianthus annuus* L.grown by pot culture method.

MATERIALS AND METHODS: In plants, assimilation of ammonia primarily takes place through Glutamine synthetase and Glutamate synthase pathway. Plants of three varieties i.e. PSH-569, PSFH-118 and KBSH-41 were raised in cemented pots and 8 kg of soil mixture was provided with metal doses Cd (II) ranging from 0, 10, 20, 50, 80 and 100 mg/kg of soil and Cr(VI) ranging from 0, 10, 20, 50, 100 and 200 mg/kg of soil mixture. Effects of Cr(VI) was observed on glutamine synthetase/glutamate synthase enzymes activity from leaves of sunflower plants at maturity, on the flowering.

Collection and processing of soil samples: The three bulk surface (0-15 cm) soil samples varying in texture and other physical characters were collected randomly, each from sand dune areas of Balsamand village, Bhadra Road, Hisar and sandy clay soil from energy park of Guru Jambheshwar University of Science & Technology, Hisar campus. The soil samples were air dried, grinded and passed through a 2 mm stainless steel sieve. The physicochemical characteristics of these soil samples were determined: pH (H_2O), organic carbon (%), electrical conductivity (EC), cation exchange capacity (CEC), total nitrogen, total phosphorus, water holding capacity, particle density and heavy metals presence etc. (Table 1 and 2)

Collection and processing of compost (Farm Yard Manure): The samples of properly decomposed farm yard manure (FYM)/compost were taken from Central Institute for Research on Buffaloes, Sirsa Road, Hisar. The physicochemical characteristics of the compost/FYM were also determined along with heavy metal analysis (Table 3), using standard methods.

Pot Culture Experiment: The sandy soil, clay soil and compost are mixed thoroughly in ratio of 1:1:1 on a plastic sheet spread over the ground. The soil mixture was divided into 108 equal lots of 8 kg each and this soil mixture was filled in 16 inches x 16 inches size polythene lined cemented pots. After 5 days, the entire contents from the pots were taken out, mixed thoroughly again and refilled in pots @ 8 kg soil mixture pot-1 and incubated for another five days.

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Table 1: Physicochemical characteristics of sandy soil, collected from sand dunes of Balsamand village, Bhadra Raod, Hisar.

Physicochemical Parameters	Values
pH*	8.2
EC (dSm^{-1})*	0.36
CEC [Cmol (P^+) kg^{-1}]	3.50
Total Organic Carbon (%)	0.12
Total Nitrogen (%)	0.23
Total Phosphorus (gkg^{-1})	0.2
$CaCO_3$ (%)	0.73
Heavy metal contents ($mgkg^{-1}$)	
Iron (Fe)	0.45
Lead (Pb)	<MDL
Cadmium (Cd)	<MDL
Chromium (Cr)	<MDL
Nickel (Ni)	<MDL
Copper (Cu)	0.027
Texture	Sand
Water Holding Capacity (%)	32
Porosity (%)	29.8 %

**1:2 Soil: Water suspension, MDL = Pb-0.06, Cd-0.01, Cr-0.05, Ni-0.03mg/l*

After filling the soil mixture, the pots were wetted with deionised water to maintain appropriate moisture content to nearly 30% and it was maintained from time to time to workable moisture level. Seeds of three varieties of Helianthus annuus L. i.e. PSH-569, PSFH-118 and KBSH-41 were washed and wetted with distilled water for 30 minutes and then treated with 0.2 % (w/v) mercuric chloride solution for 2 minutes and again washed two times with distilled H_2O, and after that five healthy seeds of three varieties were sown in each pot. Thinning was done after the emergence of seedlings and only one plant per pot was kept intact. This soil mixture was mixed with appropriate amount of 10, 20, 50, 100 and 200 mg kg^{-1} soil Cr(VI) doses separately, in solution form by using desired amount of Analytical Reagent (AR) grade $K_2Cr_2O_7$. These pots were irrigated with distilled water, as and when required, depending upon the water holding capacity of soil, so that no loss of water/minerals took place from pots. The plants were grown for 100 days to attain maturity and flowering.

Table 2: Physicochemical characteristics of soil collected from Energy Park, GJU of Sci. & Tech., Hisar.

Physicochemical Parameters	Values
pH*	8.4
EC (dSm^{-1})*	0.5
CEC (meq./100g of soil)	0.904
Total organic carbon (gkg^{-1})	31.8
Total Nitrogen (gkg^{-1})	2.4
Total Phosphorus (gkg^{-1})	0.8
Water Holding Capacity (%)	62
Bulk Density (Db)	1.33
Particle Density (Dp)	2.398
Porosity (%)	44.4
Textural class	Sandy clay
Heavy metal contents ($mgkg^{-1}$)	
Cadmium (Cd)	<MDL
Chromium (Cr)	<MDL
Copper (Cu)	<MDL
Nickel (Ni)	0.028
Zinc (Zn)	7.5

**1:2 Soil: Water suspension, MDL = Cu-0.025, Cd-0.01, Cr-0.05 mg/l.*

The metal toxicity symptoms were recorded from time to time during the plant's growth. The roots, leaves and above ground parts of harvested plants were washed with 0.1 N HCl and then with distilled water to remove dirt and dust. The washed plant material was put in paper bags, air dried first and then oven dried at 800-1100°C for 48 h.

Thereafter, dry weight of the plant materials was recorded variety wise and pot wise and various plant materials of three varieties were grinded in a stainless steel grinder and stored in polythene bags for chemical analysis. The heavy metals, Cd(II) and Cr(VI) uptake in different plant parts of each variety of sunflower was recorded, after acid digestion technique using diacid mixture of HNO_3 : $HClO_4$ (9:1) and metal analysis was done using the atomic absorption spectrophotometer (AA 6300- SHIMADZU).

On the basis of maximum heavy metal uptake, the highly Cd(II) and Cr(VI) tolerant variety of sunflower was also determined. The heavy metal accumulation in different plant parts, including root, stem, leaves, flower and seeds etc. were also determined.

Table 3: Physicochemical composition of compost (FYM) collected from C.I.R.B. Hisar.

Physicochemical Parameters	Values
pH*	7.3
Electrical conducting (EC)(dS/m)*	1.87
Total organic carbon (g/kg)	279
Total K Nitrogen (g/kg)	5.2
Phosphorus (g/kg)	7.2
Total Potassium (g/kg)	6.0
C/N ratio	53.65
C/P ratio	38.75
Heavy metal contents (mg/kg^{-1})	
Cadmium (Cd)	<MDL
Nickle (Ni)	1.61
Zinc (Zn)	23.5
Copper (Cu)	21.8
Chromium (Cr)	<MDL

For enzymatic analysis, cell free extracts were prepared in cold at 0-4°C. The tissue from metal treated leaves were macerated in extraction media in a ratio of 1 to 6 (w/v) in a chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 minutes and the supernatant collected was used as crude enzyme preparation and used for determining activities of above enzymes by dialysing the cell free extract in cold against the extraction buffer for 4h. Under the assay conditions used, the rate of enzyme catalyzed reaction was proportional to the concentration of enzyme and the reaction time (Chug, 1991).

Glutamine synthetase: The tissue from metal treated plants was homogenized in 100 mM Tris-HCl buffer (pH 7.5) with 2 mM cysteine, 2 mM $MnCl_2$, 1 mM EDTA and 10% (v/v) ethylene glycol. Activity of glutamine synthetase was estimated by both the transferase (using glutamine, hydroxylamine and ADP as substrates) and synthetic reactions (with glutamate, hydroxylamine and ATP as substrates) of the enzyme, colorimetrically by estimating the amount of γ-glutamylhydroxamate produced, according to the method given by Kanamori and Matsumoto (1972).

For the transferase activity of glutamine synthetase the reaction mixture consist of, 150µM Tris-HCl buffer (pH 7.2), 70 µM hydroxylamine hydrochloride (neutralized), 1.2 µM ADP, 80 µM sodium arsenate, 1.5 µM $MnCl_2$ and enzyme extract, in 2 ml final volume of reaction mixture. Hydroxylamine was not

added in the blanks. Incubation was done for 15 min at 270°C and reaction was stopped by adding 2 ml of the stop mixture.

The assay mixture for synthetic activity, in a final volume of 1 ml, contained 200 µM Tris-HCl buffer (pH 7.2), 10 µM glutamate, 10 µM hydroxylamine hydrochlorice (neutralized), 100 µM $MgCl_2$ and enzyme extract. In blanks hydroxylamine was not added. Reaction was stopped after incubation at 270C for 30 min by adding the stop mixture (Chug, 1991).

Estimation of γ- glutamyl hydroxamate: - Shapiro and Stadtman (1970) gave method for estimation of the amount of γ- glutamyl hydroxamate formed in the reaction mixture. 2 ml of stop mixture (4ml of 10% FeCl3, 1 ml of 24% TCA. 0.5 ml of 6N HC1 and 6.5 ml of water) was added to the reaction mixture, and centrifuged the contents at 3000 x g for 5 min. Supernatant was decanted and its optical density was recorded at 540 nm against a reagent blank. The amount of γ–glutamyl hydroxamate produced was calculated from a reference curve prepared by using authentic γ–glutamyl hydroxamate (0.2 to 1.0 µ M).

Glutamate synthase: The extraction and assay of this enzyme from sunflower leaves was given by Wallsgrove et al. (1977). Extraction was carried out in 50 mM phosphate buffer (pH 7.2) containing 12.5 mM B-mercaptoethanol, 5 mM EDTA and 1 mM PMSF. For measurement of the enzyme activity the assay mixture (0.5 ml) has 25 µmole phosphate buffer (pH 7.2); 5 µM α – ketoglutarate; 5 µmole glutamine; 100 µg methyl vialogen and dialyzed enzyme extract. Reaction was started with 50 µl of sodium dithionite solution (16 mg sodium dithionite + 16 mg $NaHCO_3$ in 0.5 ml of water) and allowed to proceed at 300C for 10 min. The reaction was stopped with 1 ml of 95% (v/v) ethanol, the contents were centrifuged and glutamate formed was separated by paper chromatographic method (Chug, 1991).

The 200 µl of the aliquot was spotted on Whatman No. 4 chromatographic paper using 20 µl of standard glutamate solution (1 mg ml-1). The chromatogram was developed in 75% phenol in presence of ammonia vapours. Dried chromatogram was then sprayed with cadmium ninhydrin reagent (At field and Morris, 1961) prepared by dissolving 0.05 g cadmium acetate in a mixture containing 5 ml of water and 1 ml of glacial acetic acid. 50 ml of acetone was added in it and precipitates formed were re-dissolved by shaking it vigorously. Finally, 500 mg ninhydrin was dissolved in this mixture. The fresh reagent was prepared every time before use.

The chromatogram was dried in an oven at room temperature in dark for 12-18 hrs in presence of 50 ml concentrated H_2SO_4.Cut out the glutamate spots from chromatogram into test tubes and the colour eluted with 8 ml of the eluting reagent (600 ml acetic acid, 600 ml of ethyl acetate, 600 ml water, 600 ml methanol, 1.8 g cadmium acetate and 18 ml glacial acetic acid) and its absorbance was read at 500 nm. Fixed amounts of glutamate were subjected to same type chromatographic treatment for preparation of the standard curve (Chug, 1991).

RESULTS AND DISCUSSION: In the data presented in Table IV the effect of chromium on production of γ glutamyl hydroxamate, revealed a very slow decline of enzyme activity in leaves of three sunflower varieties, depicting that the glutamine synthetase activity was quite insensitive to chromium treatment and was not affected by increased level of chromium in soil. The mean value of γ-glutamyl hydroxamate production was 30.1 µM min^{-1} in control, which initially increased to 30.58 and 31.34 µM min^{-1} in 10 and 20 mg kg^{-1} soil chromium level and further decreased to 29.59, 27.37 and 25.2 µM min^{-1} in 50, 100 and 200 mg kg-1 soil chromium level (Table 4). This decline in value of γ-GH production in chromium was very less showing an almost insensitive behaviour of enzyme to various chromium levels. It was found that even in the highest concentration of chromium the enzyme activity was reduced by only 17.65 and 18.22 % in PSH-569 and PSFH-118 variety and by 12.7 % in KBSH-41 variety even in 200 mg Cr kg-1 soil. The critical difference among the varieties (V=1.144) and among the initial levels (L=1.618) was found non-significant, proving that assimilation of ammonia in plants is also influenced by the activity of glutamine synthetase which in term is influenced by presence of various levels of chromium metal in the medium.

According to the Table 5, the effect of chromium on glutamate synthase activity in leaves of sunflower varieties during flowering stage depict, that mean glutamate synthase activity was 2.74 µM 30 min^{-1} relative to control, which increased to 2.89 and 2.98 µM 30 min-1 in 10 and 20 mg chromium kg^{-1} soil and then decreased to 2.67, 2.3 and 1.87µM 30 min^{-1} in 50, 100 and 200 mg kg^{-1} soil chromium level. This initial increase and further decrease in enzymatic activity revealed that a low chromium concentration (10 and 20 mg kg^{-1} soil) was favourable for enzymatic

activity, but high Cr concentration (50, 100 and 200 mg kg^{-1} soil) significantly and adversely affect the enzyme activity in leaves of three sunflower varieties. Also the maximum enzymatic activity (2.62 µM 30 min^{-1}) was observed in PSH-569 variety and minimum (2.54 µM 30 min-1) was observed in PSFH-118 variety, thus showing a greater tolerance of PSH-569 variety to chromium, relative to other two varieties. Similar results for the activity of enzyme glutamate synthase were reported by Wallsgrove et al. (1982). Suzuki and Gadal (1982) reported similar activity of glutamate synthase from leaves of Orzya sativa L. The value of critical difference was found non-significant among the varieties (V=0.118) and among the levels (L=0.167).

Table 4: Effect of chromium on glutamine synthetase activity (Production of µM γ- glutamyl hydroxamate g^{-1} fresh weight $minute^{-1}$) in leaves of *H. annuus* L. varieties on flowering stage (*Mean±SE*).

Varieties	Cr(VI) conc mg kg^{-1} soil						
	0	**10**	**20**	**50**	**100**	**200**	**MEAN**
PSH-569	30.3±1.6	30.9±1.7	31.9±0.9	30.1±0.2	27.4±0.3	24.9±0.4	29.28
% (% Change)	100 (0.0)	101.9 (+1.9)	105.24 (+5.24)	99.42 (-0.58)	90.4 (-9.60)	82.35 (-17.65)	
PSFH-118	31.0±1.0	31.3±0.6	31.7±0.6	29.3±0.6	27.5±0.4	25.4±0.3	29.4
% (% Change)	100 (0.0)	100.79 (+0.79)	101.94 (+1.94)	94.5 (-5.5)	88.43 (-11.57)	81.78 (-18.22)	
KBSH-41	28.9±1.6	29.5±1.4	30.4±1.2	29.2±0.8	27.2±0.3	25.2±0.5	28.4
% (% Change)	100 (0.0)	102.15 (+2.15)	105.31 (+5.31)	101.22 (+1.22)	94.15 (-5.85)	87.3 (-12.7)	
MEAN	30.1	30.58	31.34	29.59	27.37	25.2	

CD (P<0.05), V = 1.144, L = 1.618, V x L = 2.803

Table V:- Effect of chromium on glutamate synthase activity (Production in µM g^{-1} fresh weight $30min^{-1}$) in leaves of *H. annuus* L. varieties on flowering stage (*Mean±SE*).

Varieties	Cr(VI) conc. mg kg^{-1} soil						
	0	**10**	**20**	**50**	**100**	**200**	**MEAN**
PSH-569	2.8±0.04	2.9±0.1	3.0±0.04	2.7±0.09	2.3±0.05	1.9±0.04	2.62
% (% Change)	100 (0.0)	104.04 (+4.04)	107.37 (+7.37)	97.5 (-2.5)	83.23 (-16.77)	69.32 (-30.68)	
PSFH-118	2.6±0.2	2.8±0.07	2.9±0.12	2.6±0.08	2.2±0.06	1.8±0.04	2.54
% (% Change)	100 (0.0)	110.03 (+10.03)	112.44 (+12.44)	101.02 (+1.02)	87.06 (-12.94)	70.05 (-29.95)	
KBSH-41	2.7±0.22	2.8±0.16	2.9±0.04	2.6±0.09	2.2±0.05	1.8±0.03	2.55
% (% Change)	100 (0.0)	102.64 (+2.64)	106.84 (+6.84)	94.25 (-5.75)	81.54 (-18.46)	65.11 (-34.89)	
MEAN	2.74	2.89	2.98	2.67	2.3	1.87	

CD (P<0.05), V = 0.118, L = 0.167, V x L = 0.29

CONCLUSION: From the findings of above research work it is concluded that hexavalent chromium despite being toxic to plant metabolism, not be able to show its deleterious effects to glutamine synthetase activity upto concentration of 100 mg $kg.^{-1}$ experimental soil. However at 200 mg $kg.^{-1}$ experimental soil, it reduces the enzyme activity by 18 % only. For glutamate synthase enzyme activity, the lower concentration of 10 mg – 20 mg $kg.^{-1}$ soil is not harmful but a concentration above this is harmful in experimental conditions for sunflower plants.

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