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Cadmium Treatment Alters Phytochemical and Biochemical Activity in *Glycine max* L.

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Abstract: *Glycine max* L. (Soybean) is known for having its medicinal and nutritional value. It has capacity to accumulate high concentrations of cadmium (Cd). Studies were carried out to evaluate secondary metabolites production and biochemical potential of plant exposed to Cd-rich growing medium. Cd treatment in the form of Cadmium chloride ($CdCl_2$) at supply of 0.25, 0.50, 1.00 and 2 mg L^{-1} increased the amino acid, protein, proline and diadzene content in field grown soybean plants at different developmental stages of *Glycine max* L. The enhancement was significant at Cd treatment of 1 mg L^{-1}. The activity of antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT) and Glutathione Reductase (GR) increased with increase in treatment of Cd in all developmental stages of soybean but increase in enzyme activity was more significant at Cd treatment of 2 mg L^{-1}. Present finding suggested that higher diadzene production in cd enriched soyabeen contributed to higher medicinal value.

Key words: Soybean, cadmium, diadzene, oxidative stress

INTRODUCTION

Active industrialization have released biologically significant amount of heavy metals into our environment. Heavy metal concentration has increased in soil, surface water and posed potential threat to terrestrial and aquatic biota (Nasim and Dhir, 2010). Contamination of medicinal plants with heavy metals pose serious problem to quality of medicinal plants and efficacy of their products (Lizhong and Cullen, 1995; Ajasa *et al*., 2004; Nasim and Dhir, 2010). Heavy metal such as cadmium (Cd) has been widely used in many industrial processes and it has a long biological half-life. It is a nonessential heavy metal that does not have any metabolic function in higher plants. Under natural Conditions, it exists at low concentrations in most soils. It has a great mobility in the soil as compared with other heavy metals and is taken up in varying concentration by plants (Varo *et al*., 1980). The increasing amount of Cd in the environment affects growth and developmental events in plant and animals (Di Toppi and Gabbrielli, 1999; Shanker *et al*., 2005; Dhir *et al*., 2008). At the cellular level Cd induced changes in lipid composition, the activity of enzymes associated with membrane and the distribution of macro and micronutrients in plants (Nasim and Dhir, 2010).

The secondary metabolism is essential for the fitness of plants; many secondary metabolites play important function as chemical defense compounds against herbivores, microbes or competing plants (Zheng and Wu, 2004). Due to the complexity and flexibility of secondary metabolism, as well as our limited understanding of the mechanism connecting primary and secondary metabolism, comprehensive studies on the effect of heavy metals on the production of secondary metabolites are insufficient. Though extensive literature is available focusing the impact of Cd supply on crop plants but studies related to effects on potential medicinal plants and their properties are not sufficient. So, it was essential to examine the effect of Cd on medicinal plant and their metabolites production and other associated aspects (like amino acid, protein, proline and antioxidant enzyme) in the plant. Present investigations were carried out with an aim to: (1) evaluate and compare the production of diadzene (metabolite) in Cd treated plants of soybean at different developmental stages (2) analyze effect of Cd on protein, amino acid and diadzene contents in soybean at different developmental stages and (3) evaluate effect of Cd on activity of antioxidant enzymes in different developmental stages of plant growth.

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MATERIALS AND METHODS

Plant material: Seeds of local Indian *Glycine max.* L. (Family-leguminosea) were obtained from Indian Agricultural Research Institute IARI (New Delhi) was used for the present study. Plant identification was confirmed and voucher specimen (PK 1042) was deposited at Jamia Hamdard, New Delhi, India.

Experimental set up: Field experiment was conducted in the kharif season at the experimental field of Hamdard University, New Delhi. The individual plot size was 6 m^{-2} (4×1.5 m) having 6 rows with a row to row distance of 15 cm and plant to plant distance of 10 cm. The numbers of plants per m^2 were 15. Before sowing, seeds of soybean were surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with double distilled water. Before seed sowing, Cd was applied at the rates of 0.25, 0.50, 1.00 and 2.00 mg L^{-1} from $CdCl_2$. The experiment was conducted according to a simple randomized complete block design. Each treatment was replicated five times. The plots were watered as and when required and plants were grown under naturally illuminated environmental conditions.

Cadmium treatments: The soybean plants were exposed to different concentrations of Cadmium chloride (0.25, 0.5, 1.00, 2.00 mg L^{-1}) under field growing condition. The plants were uprooted at three different developmental stages (i.e. Pre-flowering, flowering and post flowering) for measuring the amino acid, protein, proline and enzyme activity after Cd treated condition. The collected sample at three different developmental stages were washed in demineralized water and the plant material was dried in a mechanical convection oven at 75°C, until constant mass, for estimation of bioactive production.

Estimation of protein: The 0.5 g plant material was ground in prechilled mortar and pestle with 1.0 mL (0.1M) phosphate buffer (pH 7.0) on ice and centrifuged at 5,000 rpm for 10 min at 4°C. TCA (0.5 mL) was added and the sample was again centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 1.0 mL of 0.1N NaOH after washing with double- distilled water. After adding 5.0 mL of Bradford reagent, the optical density was measured at 595 nm (Bradford, 1976).

Estimation of free amino acids: Free amino acids content was estimated by the method of Lee and Takahashi (1966). In brief, 0.1 g plant material was incubated overnight in 70% ethanol followed by washing with double-distilled water. Then, 1.5 mL of 55% glycerol and 0.5 mL ninhydrin solution were added, boiled at 100°C for 20 min and cooled down. The final volume was made up to 6 mL with double-distilled water and the optical density was measured at 570 nm.

Proline determination: The amount of proline was determined according to the method described by Bates *et al.* (1973). 0.3 g of sample from control and treated plants were homogenized by the addition of 5 mL of 3% sulphosalicylic acid solution. The materials were homogenized by using a cold mortar and pestle. The homogenate was centrifuged at 5000 g for 10 min at 4°C. Two millitter of acid ninhydrin (0.31 g ninhydrin, 7.5 mL of acetic acid and 5 mL of 6M phosphoric acid), 2 mL of 96% acetic acid and 1 mL of 3% sulphosalicylic acid were prepared and supernatant (2 mL) from each homogenate was added to the tubes. The tubes were incubated at 96°C for 1 h in a hot water bath and after incubation 4 mL of toluene was added to each tube followed by mixing. The absorbance of the pink red color upper phase was recorded at 520 nm against toluene blank. A standard curve for proline in the range of 0.01 to 1.5 mM was constructed to determine the proline concentration in each sample.

Determination of enzyme activities: The activities of SOD, CAT and GR were measured according to the method described by Misra and Gupta (2006). For SOD, the assay buffer consisted of 20 mM sodium Phosphate (pH 7.5), 0.1 mM EDTA, 10 mM methionine, 0.1 mM p-Nitro Blue Tetrazolium chloride (NBT), 0.005 mM riboflavin and the enzyme extract containing 50 mg protein in a final volume of 3 mL. SOD activity was determined by the inhibition of NBT photo reduction. A fluorescent lamp was positioned at a distance of 20 cm from the samples for 5 min. One unit of SOD was defined as the amount of enzyme that inhibits NBT photo reduction by 50% when monitored at 560 nm. A standard curve for SOD in the range of 20 to 200 ng mL^{-1} was constructed to determine the SOD concentration in each sample.

For CAT, the assay medium consisted of 20 mM sodium phosphate buffer (pH 7.5), 6 mM H_2O_2 and crude extract containing 3 mg/mL proteins in a final volume of 1 mL. The reaction was initiated by the addition of H_2O_2. The decrease in the absorbance of H_2O_2 was recorded at 240 nm for 3 min. The enzyme activity was calculated as the amount of H_2O_2 consumed using the extinction coefficient of H_2O_2 (40 $mM^{-1}cm^{-1}$at 240 nm).

For GR, the assay mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM Na_2EDTA, 0.1 mM nicotinamide adenine dinucleotide phosphate

(NADPH), 1.0 mM oxidized glutathione (GSSG) and the enzyme extract containing 5 mg protein in a final volume of 1 mL. The decrease in the NADPH concentration was recorded at 340 nm.

Extraction and quantification of diadzene compound: Diadzene was extracted from plant material following modified procedure as described by Heimler *et al.* (2004). One hundred milligram of harvested sample was used for extraction. The extraction was carried out at room temperature using 70 mL Ethanol-water (70:30, v/v) at pH<2. The raw ethanolic extracts were defatted with 15 mL of n-hexan. The ethanolic extracts were then evaporated to dryness under vacuum at room temperature and finally rinsed with ethanol/water (70/30, pH 2.0) to a final volume of 1.5 mL. Samples of 4 μL were analyzed by HPLC for quantitative evaluation. Preparation of diluted standard Diadzene was done following method of Heimler *et al.* (2004).

Statistical analysis: The data obtained was expressed as Mean±standard error. The mean values obtained from five replicates from three independent experiments were checked for the level of significance using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Cadmium treatment increased diadzene (bioactive) production at different developmental stages of soybean except at concentration of 2 mg L^{-1} (Table 1). The concentrations of diadzene were significantly higher in all stages of soybean up to 1 mg L^{-1}. Earlier studies suggested that heavy metals may play an important role in triggering plant genes to alter the titers or nature of secondary plant metabolites, although the exact mechanism by which this happens remains unclear (Nasim and Dhir, 2010). Oxidative stress induced by heavy metals triggers signaling pathways that affect production of specific plant metabolites (Nasim and Dhir, 2010). Similar observations have been noted by several research groups (Michalak, 2006; Eman *et al.*, 2007; Nasim and Dhir, 2010). However, high levels of heavy metal contamination in medicinal or other plants may suppress secondary metabolite production as in our studies (Table 1). Alternatively, the presence of heavy metals in medicinal plants may stimulate production of bioactive compounds in many plant species.

The result indicated that Cd treatment did not increase significantly in amino acid and protein contents in all stages of seedlings up to 1 mg L^{-1} Cd treatment and decreased thereafter (Table 2). Protein synthesis was greatly affected by Cd treatments. There was a considerable difference in plants protein content between plants treated with Cd and control. The reduction in the amount of protein could be due to decrease in protein synthesis or an increase in the rate of protein degradation (Balestrasse *et al.*, 2003). Earlier work reported that heavy metal-induced changes in plant growth patterns and metabolic activities affect production of amino acid, proteins, photosynthetic pigments, sugars and non-protein thiols. Such effects may result from inhibition of various enzymes involved in biosynthesis of these natural products or, more likely, through impaired substrate utilization (Singh *et al.*, 2006; Kovacik *et al.*, 2006; Rai and Mehrotra, 2008). Further, Palma *et al.* (2002) stated that under stress condition the decrease in protein content in plants may be caused by enhanced protein degradation process as a result of increased protease activity. It is also likely that these heavy metals may have induced lipid per oxidation in plant and fragmentation of proteins due to toxic effects of reactive oxygen species

Table 1: Diadzene content (μg g^{-1} dry weight) of soybean at various developmental stages under control and cadmium treated condition

Cadmium treatments (mg L^{-1})	Pre-flowering	Flowering	Post-flowering
0.00	1.19±0.07^{ab}	1.23±0.11^{ab}	1.12±0.09^{ab}
0.25	1.73±0.12^{b}	1.91±0.10^{b}	1.57±0.11^{b}
0.50	1.98±0.13^{b}	2.12±0.23^{b}	1.93±0.15^{b}
1.00	2.80±0.31^{a}	2.93±0.19^{a}	2.30±0.17^{a}
2.00	2.58±0.52^{a}	2.59±0.33^{a}	2.13±0.18^{a}

Within each column, values followed by the same superscript letters are not significantly different at $p \leq 0.05$ level

Table 2: Protein, amino acid, Proline contents (μg g^{-1} dry weight) of soybean at different developmental stages under control and cadmium treated conditions

	Pre-flowering			Flowering			Post-flowering		
Cadmium treatments (mg mL^{-1})	Protein	Amino acid	Proline	Protein	Amino acid	Proline	Protein	Amino acid	Proline
0.00	3.52±1.09^{b}	2.44 ±0.91^{b}	1.31±0.18^{c}	3.88±0.09^{b}	2.98±0.89^{b}	1.38±0.09^{c}	3.44±0.09^{b}	2.38±0.09^{b}	1.46±0.09^{c}
0.250.25	3.23±0.98^{b}	2.33±0.47^{b}	1.59±0.23^{bc}	3.75±0.09^{b}	2.87±0.71^{b}	1.63±0.09^{b}	3.18±0.09^{b}	2.15±0.09^{b}	1.98±0.09^{b}
0.50	2.81±1.06^{b}	2.28±0.33^{b}	1.93±0.48^{b}	3.19±0.09^{b}	2.68±0.63^{b}	1.98±0.09^{b}	2.48±0.09^{ab}	1.98±0.09^{b}	2.10±0.09^{b}
1.00	1.99±1.09^{a}	1.83±0.51^{a}	2.33±0.37^{a}	2.05±0.09^{a}	2.21±0.68^{a}	2.57±0.09^{a}	1.38±0.09^{a}	1.53±0.09^{a}	2.88±0.09^{a}
2.00	1.24±0.54^{a}	1.51±0.30^{a}	2.99±0.76^{a}	1.88±0.09^{a}	1.99±0.47^{a}	3.18±0.09^{a}	1.07±0.09^{a}	1.21±0.09^{a}	3.41±0.09^{a}

Within each column, values followed by the same superscript letters are not significantly different at $p \leq 0.05$ level

Table 3: SOD, GR and CAT activity (unit mg^{-1} protein) of soybean at various developmental stages under control and cadmium treated condition

Cadmium treatments	Pre-Flowering			Flowering			Post flowering		
(mg L^{-1})	SOD	GR	CAT	SOD	GR	CAT	SO	DGR	CAT
0.0	031.95±2.09^{e}	7.44±1.11^{c}	4.80±0.19^{c}	43.98±2.02^{c}	7.98±1.19^{c}	5.21±1.01^{c}	51.98±1.09^{c}	8.51±1.19	°5.90±1.09^{c}
0.25	38.41±2.10^{bc}	9.41±1.10^{bc}	5.13±0.73^{bc}	47.18±2.09^{bc}	10.11±1.09^{bc}	5.88±1.21^{bc}	58.18±1.89^{bc}	11.98±0.99^{bc}	6.18±1.29^{bc}
0.50	45.11±2.16^{b}	12.87±2.03^{b}	5.79±0.87^{b}	53.13±2.10^{b}	13.18±1.07^{b}	6.20±1.07^{b}	66.11±1.19^{b}	14.99±1.11^{b}	6.98±1.44^{b}
1.00	51.23±2.31^{b}	14.49±2.11^{b}	6.33±0.98^{b}	61.23±1.91^{b}	16.00±1.59^{b}	6.78±1.11^{b}	71.55±1.19^{b}	17.08±1.09^{b}	7.24±1.34^{b}
2.00	63.34±2.04^{a}	19.24±1.91^{a}	7.41±0.66^{a}	68.21±2.09^{a}	21.18±2.09^{a}	7.98±1.19^{a}	77.43±2.19^{a}	22.18±2.09^{a}	8.18±1.79^{a}

Within each column, values followed by the same superscript letters are not significantly different at $p \leq 0.05$ level

led to reduced protein content (Davies *et al*., 1987). Our studies also coincide with Costa and Spitz (1997) who also reported a decrease in amino acid and soluble protein content under heavy metal stress in *Lupinus albus*.

Comparison of proline contents in different stages of plant growth are shown in Table 2. Proline contents noted increase in Cd treated soybean plants but results were significantly higher at 2 mg L^{-1} Cd treatment (Table 2). Proline has been shown to play an important role in recovering from environmental stresses in plants and its accumulation might be induced as a result of Reactive Oxygen Species (ROS). The mechanisms by which proline reduces oxidative damage include physical quenching of singlet oxygen and chemical reaction with hydroxyl radicals (Alia and Matysik, 2001). Due to the chelating ability, proline can also be a defense mechanism for survival of stressed plants by binding with metal ions. Proline accumulation in shoots of *Brassica juncea*, *Triticum aestivum* and *Vigna radiata* in response to Cd toxicity was demonstrated by Dhir *et al*. (2004) but they also found that proline accumulation decreased with the exposure to Cd in hydrophytes (*Ceratophyllum*, *Wolffia* and *Hydrilla)*.

Enzymatic antioxidant system is one of the ways to resist the oxidative damage caused by abiotic stress (Mittler, 2002). Enzymes such as SOD, CAT, GR have a physiological function under non-stressed conditions, but their activity or quantity is increased under oxidative damage. However, the role of antioxidant systems in detoxification of Cd is still not clear. Activity of CAT noted an increase with Cd treatment but the highest activity was found at 2 mg L^{-1} Cd treatments (Table 3). Our results demonstrated that Cd toxicity mediated changes in the activities of antioxidant enzymes are dose dependent. Increasing the activities of CAT which are scavengers of H_2O_2, might result in reduced formation of superoxide anion radicals (Chen *et al*., 2003). Higher Cd levels were also reported to induce the activity of CAT in *Spirulina platensis* (Mittler, 2002).

CONCLUSIONS

This study points out that the bioactive (i.e., diadzene) production varies in a concentration and duration-dependent manner. Cd supply in growing medium enhanced production of metabolite diadzene. The present studies can be useful for developing a protocol with a prospect of getting high diadzene yield by altering growing conditions. Besides this, more studies need to be carried out to understand the mechanism involved and evaluate the factors responsible for such enhancement.

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