



# **Influence of Chromium Stress on Chlorophyll Content, Polyphenol Oxidase and Peroxidase Activity in Horse Gram (*Dolichos biflorus* L)**

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## **Authors' contributions**

*This work was carried out in the corresponding author's laboratory. Author KJ is a research scholar under the supervision of the corresponding author. Both authors read and approved the final manuscript.*

## **Article Information**

DOI: 10.9734/EJMP/2022/v33i130442

## **Open Peer Review History:**

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/82146>

**Original Research Article**

**Received 02 November 2021**

**Accepted 04 January 2022**

**Published 05 January 2022**

## **ABSTRACT**

The presence of heavy metals in solid and liquid wastes is a significant issue in terms of the environment degradation. These are one of the most serious environmental pollutants, and reaching dangerous levels will need more investigation. Chromium, in particular, has become a global environmental problem among heavy metals. This research looked at the effects of Cr<sub>2</sub>O<sub>7</sub> stress on *Dolichos biflorus* L., a kind of horse grain that plays an essential role in Indian agriculture. *D. biflorus* seeds were cultivated in the dark under laboratory conditions with a Sodium chromate concentration of (0-3.0mg/L). The control treatment was distilled water. Seven-day-old seedlings were utilized to study the effects of chromate stress on peroxidase activity and chlorophyll content. The findings showed that when the quantity of Sodium chromate increased, the chlorophyll content of *D. biflorus* seedlings increased considerably (p 0.9). Increased polyphenyloxiase and peroxidase activity indicated the appearance of a scavenging mechanism in plants under heavy metal stress, whereas increased peroxidase quantity indicated the generation of free radicals. The drop in chlorophyll concentration indicates that the plants' development has slowed, resulting in a fall in production.

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**Keywords:** Chlorophyll; heavy metal stress; sodium chromate; polyphenyloxidase; peroxidase; seedling: *D. biflorus* etc.

## 1. INTRODUCTION

The industrialization and urbanization during the past few years have given rise to serious problems of environmental pollution. General increases in the level of heavy metals pose a pervasive threat to the natural ecosystem [1]. The presence of heavy metals in the environment is a serious ecological problem, because these elements can enter food chains and the biological cycle [2]. The plants under stress conditions are most likely to be adversely affected by high concentration of heavy metals. The possible adverse effects of heavy metal pollution and their phytotoxic effects have been reported in *Shorea robusta* [3], *Capsicum annum* [4], *Albizia procera* [5].

Among the metals, lead particularly has become a cosmopolitan environmental pollutant [6]. Chromium is a toxic environmental contaminant that induces many biochemical and structural changes in biological systems [7]. The process of photosynthesis is adversely affected by Chromium toxicity. One of the phytotoxic effects of Chromium appears to be induction of oxidative stress in growing plant parts due to enhanced production of reactive oxygen species (ROS) resulting in an unbalanced cellular redox status. A number of different ROS, including the superoxide anion ( $O_2^-$ ), singlet oxygen ( $O_2$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical (OH) are produced during normal oxidative metabolism in aerobic organisms, but these ROS can pose a severe threat when produced in larger amounts Sharma and Dubey [6]. Although the ROS generating processes are slow under normal conditions, Chromium accelerates them Verma and Dubai [8].

It is the goal of this study to evaluate the effects of various sodium chromium concentrations on the chlorophyll content, lipid peroxidation, and peroxidase activity of the plant *D. biflorus*. With this newfound understanding of the physiological and biochemical foundation of phytotoxicity, it will be easier to identify potential restrictions in the function of lead pollutants in plants. Material and Methods of Construction Agricultural University Hyderabad provided the seeds for the *D. biflorus* that was employed in the current investigation.

It is expected that the present study effort will be completed at the Department of Botany at

Osmania University College in Hyderabad (T.S.) between December 2018 and May 2020. Seeds of uniform size were selected and surface sterilized with a % solution of mercuric chloride for 5 minutes to prevent fungal growth, after that they were rinsed 4-5 times with water in order to remove any residual mercuric chloride [9]. Next, 50 seeds were placed in 10 cm diameter Petri plates coated with Whatman No. 1 filter paper moistened with distilled water for 24 hours in the dark to see if these did germinate.

## 2. MATERIALS AND METHODS

Extraction and estimation of total chlorophylls and carotenoids were done by the method of Lichtenthaler [10]. 1 gm of fresh leaf material was taken into a mortar and the tissue ground to a fine pulp with 40 ml of 100% acetone. Resulted green liquid was transferred to a Buchner funnel containing a pad of Whatman no.1 filter paper. The grinding was repeated till the tissue got devoid of any pigments with 100% acetone. Mortar and sides of the funnel were rinsed with 10 ml of 100% acetone to ensure that all the pigments are collected. Final volume was adjusted to 100 ml with acetone.

The optical densities of pigment extracts were read at 661, 654 and 470 nm in Shimadzu-160A-UV- Visible Recording Spectrophotometer. The absorbance was measured against 100% acetone as blank. The pigment concentration was calculated using the following formula.

The optical density of extract was measured at 661, 644 and 470nm. The pigment concentration was calculated as follows and expressed as mg gm<sup>-1</sup> fresh weight of leaf.

$$\begin{aligned} C_a &= 11.24 A_{661} - 2.04 A_{644} \\ C_b &= 20.13 A_{644} - 4.19 A_{661} \\ C_{a+b} &= 7.05 A_{661} + 18.09 A_{644} \end{aligned}$$

$$C_{x+c} = \frac{1000A_{470} - 1.90 C_a - 63.14 C_b}{214}$$

Where,

$C_a$  = concentration of chlorophyll a (mg g<sup>-1</sup> Fr. Wt.)

$C_b$  = concentration of chlorophyll b (mg g<sup>-1</sup> Fr. Wt.)

$C_{a+b}$  = concentration of total chlorophylls (mg. g<sup>-1</sup> Fr. Wt.)

$C_{x+c}$  = concentration of total carotenoids (xanthophyll and carotene, mg. g<sup>-1</sup> Fr. Wt.)

A = absorbance measurement at wave length

Peroxidase activity was estimated as per the method of Kar and Mishra (1976). The reaction mixture consisted of 2 ml of Tris-HCl buffer 0.1M (pH 7.0), 1 ml of pyrogallol (0.01M), 1 ml of H<sub>2</sub>O<sub>2</sub> (0.05M) and 1 ml enzyme extract. A blank was prepared simultaneously with 3 ml of Tris-HCl buffer, 1 ml of 0.05M H<sub>2</sub>O<sub>2</sub> and 1 ml of enzyme extract. The reaction mixture was incubated at 25°C for 5 minutes. The reaction was stopped by adding 1 ml of 2.5N H<sub>2</sub>SO<sub>4</sub>. The amount of purpurogallin formed was estimated by measuring the absorbance at 425 nm in a Shimadzu UV- Visible spectrophotometer. The enzyme activity was expressed as change in absorbance units.

### 3. RESULTS AND DISCUSSION

Effect of Sodium chromate on content of chloroplast pigments in *D. biflorus*. Plants are shown in (Table 1), (Fig 1), (Plate 1).

Maximum amount of chl. a (3.21 mg), chl. b (1.80 mg) and total chlorophylls (5.03 mg) were found in control plants; whereas in (3.0 mg/ml) of Sodium chromate treated *D. biflorus* plants, lower contents of chl. a (2.20 mg), chl. b (1.21 mg) and total chlorophylls (3.41 mg) were observed, indicating a reduction of 68 % of chl. a, 67 % of chl. b and 67 % of total chlorophylls. With the increase in the conc. of Sodium chromate there was a reduction in the levels of chloroplast pigments.

The quantity of carotenoids is more (23.05 mg) in control plants as compared to Sodium chromate treated plants (12.23mg), indicating a decrease of 53.05 %. Lower conc. of Sodium chromate did not affect the number of carotenoids. Decrease in the content of chlorophyll pigments due to Sodium chromate treatment was reported earlier by Sunitha and Rathore [11] in *Vicia faba* and *Allium cepa*., Gritsam et al. [12] in *Robinia pseudoacacia* (L), Gangdhar Rao et al., [13] in *Raphanus sativus* and Ballantyne [14] in *Rhododendron*.

Hovarth et al. [15] suggested that the reduction in content of chlorophyll pigments might be due to destruction of chloroplast by fluoride injury. According to Mikhailova et al. [16] hydrogen fluoride has the greatest impact on the cells of the mesophyll thereby decreasing chlorophyll

pigments. Gangadhar Rao et.al [13] studies revealed that fluoride caused more injury in radish leaves and decreased the content of chlorophylls in the absence of calcium, magnesium and phosphorus in the growth medium. Studies by Treshow [17] revealed that fluoride may affect early stages of pigment synthesis or induces the degradation of chloroplast structure.

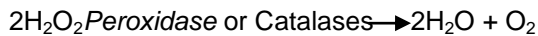
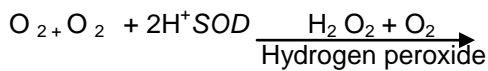
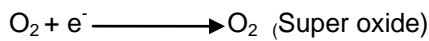
A perusal of the available literature reveals that so far, no work has been done on the impact of Sodium chromate treatment on carotenoids. Effect of Sodium chromate on the activity of super oxide dismutase (SOD) in *D. biflorus* represented in (Table-2 and Fig-2).

The activity of SOD increased from 43.05 enzyme units in control to 76.00 enzyme units in Sodium chromate treated plants. With the increase in conc. of Sodium chromate there was an increase in the activity of SOD. About 75 % increase in the activity of SOD was observed in plants treated with 3.0 mg/ml, Sodium chromate. Superoxide dismutase (SOD) is a protective antioxidant enzyme. It helps in preventing both direct toxicity from superoxide free radicals (O<sub>2</sub><sup>-</sup>) and secondary toxicity from OH and H<sub>2</sub>O<sub>2</sub> [18]. Free radicals can occur in both organic and inorganic molecules. They are highly reactive and therefore highly unstable and short-lived [19]. Free radicals are derived from both natural and anthropogenic sources. They are produced naturally *in vivo* as by products during normal metabolism. Example include superoxide free radical (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub>. Production of reactive oxygen species such as O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> is an unavoidable consequence of aerobic metabolism. In plants, mitochondrial electron transport chain (ETC) is a major site of superoxide free radical production. Anthropogenic sources of free radical production are ionizing radiation, certain drugs or environmental pollutants such as NO<sub>2</sub>, CN, herbicides and various xenobiotics. It is a well-established fact that fluoride causes the production of free radical [20].

Free radicals can cause chain reactions and damage critical cellular constituents such as proteins, lipids and DNA. In proteins consequence of free radical attacks include peptide chain scission and denaturation, while in DNA, strand scission or base denaturation [21,22,23]. Superoxide radicals can also cause lipid peroxidation in plasma membrane and membranes of organelles. Interaction with other

cellular constituents can also occurs resulting in damage.

Superoxide radicals must be detoxified as effectively as possible to minimize damage. This constitutes a defense mechanism against detrimental effects of superoxide radicals. Therefore living systems have evolved an intracellular enzymatic defense system to protect themselves against superoxide radicals in the form of superoxide dismutase.



Over expression of mitochondrial Mn-SOD in mammalian cell show increased resistance to  $O_2^-$  generating reagent paraquat. When plant mitochondrial Mn-SOD was expressed in the mitochondria of a Mn-SOD deficient yeast strain, the cells regained their resistance to oxidative stress. There are various types of isoenzymes in their requirement for detoxification  $O_2^-$  viz: Cu-Zn-SOD, Mn-SOD and Fe-SOD.

The activity of SOD is increased from 43.05 enzyme units in control to 76.00 enzyme units in Sodium chromate treated plants. With the increase of Sodium chromate concentration there was increase in activity of SOD. About 45 % increase in the activity of SOD was observed in 2.0 mg/ml Sodium chromate treated plants.

Table-1 represents the effect of sodium chromate on the concentration of chloroplast pigments in *D. biflorus* plants (Fig. 1). In control plants, the

maximum concentrations of total chlorophylls, Chlorophyll a, and Chlorophyll b are observed, whereas the concentrations were greatly decreased in plants treated with 2 mM of Sodium Chromate. Concentration of Sodium Chromate has led to the reduction in chlorophyll a, chlorophyll b, and total chlorophyll content. In control plants, the concentration of carotenoids was higher, whereas in plants treated with Sodium Chromate, the concentration of carotenoids dropped. Very little change in the amount of carotenoids occurred when the sodium chromate was at a lower concentration.

Sunitha and Rathore [11] observed decreased content of chlorophylls in *Vicia faba* and *Allium cepa*. Gritsamet al. [12] discovered decreased content of chlorophylls in *Robinia pseudoacacia* (L). Gangadhar Rao et al. [13] noted decreased content of chlorophylls in *Raphanus sativus*, and Ballantyne [14] reported decreased content of chlorophylls in Rhododendron.

According to Hovarth et al., [15], it is likely that chloroplast is destroyed owing to fluoride damage, as a result of which Chlorophyll pigments are lost. The work of Mikhailova et al., [16] found that hydrogen chromate and fluoride had the largest effect on the mesophyll, resulting in lower chlorophyll pigment concentrations in the cells. In a study published in 1995, Gangadhar Rao et al., [13] showed that fluoride had a greater impact on radish leaves and that this occurred without the usage of any other minerals, including calcium, magnesium, and phosphorus. Fluoride may influence the early phases of pigment production, as shown by Treshow [17], or might damage the chloroplast structure. The carotenoids have not been investigated in relation to chromate toxicity.

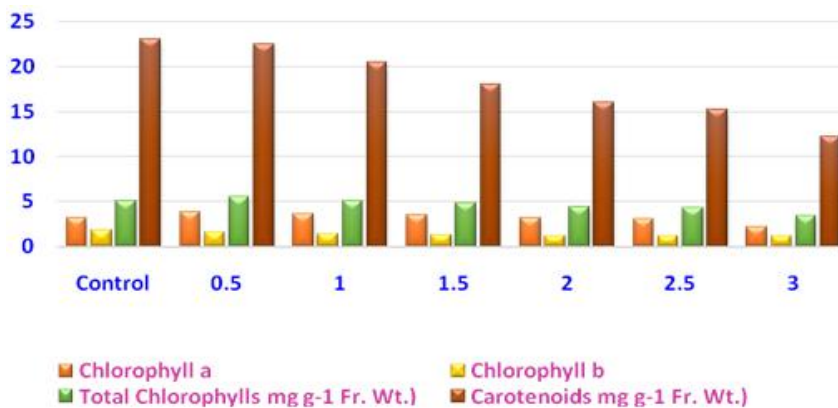


Fig. 1. Effect of sodium chromate on content of chlorophyll pigment (mg g<sup>-1</sup> Fr. Wt.) in *D. biflorus*

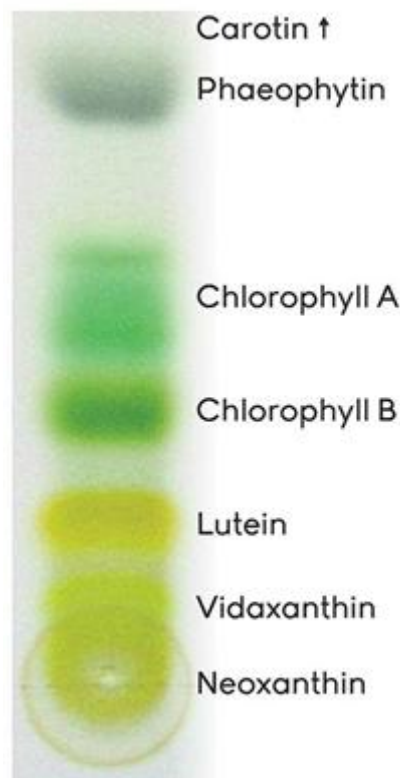
**Table 1. Effects of different concentrations of sodium chromate on content of chlorophyll pigment (mg g<sup>-1</sup> Fr. Wt.) in *D. biflorus***

Concentrations of Na <sub>2</sub> Cro <sub>4</sub> mg/ml	Chlorophyll a (mg g <sup>-1</sup> Fr. Wt.) ±(SE)*	Chlorophyll b (mg g <sup>-1</sup> Fr. Wt.) ±(SE)*	Total Chlorophylls (mg g <sup>-1</sup> Fr. Wt.) ±(SE)*	Carotenoids (mg g <sup>-1</sup> Fr. Wt.) ±(SE)*
Control	3.23 ± 0.23	1.80 ± 0.12	5.03 ± 0.35	23.05 ± 0.32
0.5	3.83 ± 0.14	1.64 ± 0.13	5.47 ± 0.44	22.50 ± 0.22
1.0	3.62 ± 0.32	1.43 ± 0.12	5.05 ± 0.44	20.50 ± 0.12
1.5	3.52 ± 0.24	1.32 ± 0.14	4.84 ± 0.38	18.02 ± 0.93
2.0	3.21 ± 0.53	1.23 ± 0.14	4.44 ± 0.67	16.03 ± 0.32
2.5	3.10 ± 0.23	1.22 ± 0.12	4.32 ± 0.35	15.23 ± 0.24
3.0	2.20 ± 0.21	1.21 ± 0.12	3.41 ± 0.33	12.23 ± 0.32

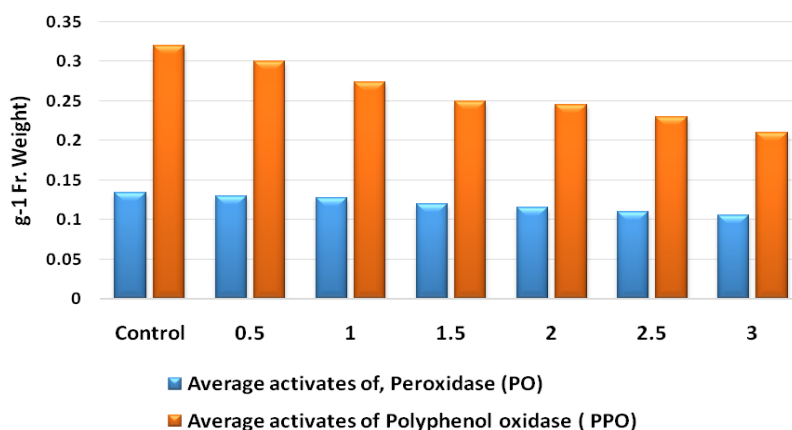
Values represent means ± Standard error of 3 replicates per treatment

**Table 2. Effect of different concentration of sodium chromate on the activities of Catalase, Peroxidase and Polyphenol oxidase on *D. biflorus***

Concentrations of Na <sub>2</sub> Cro <sub>4</sub> mg/ml	Average activates of, Peroxidase ± (SE)*	Average activates of Catalase ± (SE)*	Average activates of Polyphenol oxidase ± (SE)*
Control	0.134 ± 0.20	232.00 ± 0.12	0.320 ± 0.20
0.5	0.130 ± 0.02	245.02 ± 0.42	0.300 ± 0.03
1.0	0.128 ± 0.03	254.02 ± 0.02	0.274 ± 0.03
1.5	0.120 ± 0.20	260.45 ± 0.32	0.250 ± 0.02
2.0	0.115 ± 0.02	280.00 ± 0.32	0.245 ± 0.20
2.5	0.110 ± 0.05	290.00 ± 0.02	0.230 ± 0.05
3.0	0.105 ± 0.02	320.00 ± 0.02	0.210 ± 0.04



**Plate 1. Thin layer chromatographic separation (TLC); after one-year-old plant leaves**



**Fig. 2. Effect of sodium chromate on the activities of peroxidase (PO) and Poyphenol oxidase (PPo) in (g-1 Fr. Weight) on *D. biflorus***

Effect of Sodium Chromate on the activities of peroxidase and catalase in *D. biflorus* plant is represented in (Table-2) (Fig. 2). Sodium Chromate inhibited the activities of Polyphenol oxidase and Peroxidase. Maximum activities of these two enzymes (PPO 0.320 units and PO 0.134 units) were observed in control plants and in treated plants their enzyme activities were decreased (to 0.210 units in PPO and 0.105 units in P.O.). Whereas, the activity of catalase was increased from 232 units in control to 320 units in treated plants. Sensitivity of several enzymes to fluoride is influenced by many factors Mac Lean, et al., [24].

Decrease in the activities of PPO & PO can be attributed to sensitivities of these enzymes to sodium chromate. Similar observations were reported by Lee *et al*, (1966) in chromate fumigated Soya bean leaves. Decrease in the activities of P.O. and P.P.O. was seen in Sodium NaF treated Radish leaves by Gangadhar Rao, et al, [13]. Increase in catalase activity in Sodium Chromate treated *D. biflorus* plants may be taken as a response to toxicity of chromate as a stress factor. These results are in agreement with Lee et al, (1966) and Gangadhar Rao, et al [13].

#### 4. CONCLUSION

From the observed results, it is concluded that the activity of polyphenyloxidase, peroxidase increased in response to the increased metal ion concentration from 0.5mg/L to 3.0mg/L in *D. biflorus*. The increased polyphenyloxidase, content indicates the production of free oxygen radical, whereas the increased peroxidase activity indicates the scavenging mechanism of the plants against the ROS produced in response to the metal stress. The chlorophyll content of the

metal stressed leaves decreased with increased concentration of metal ion from 2.0 mg/L to 3.0mg/L. The decreased chlorophyll content is a visible symptom of reduced growth. The defense mechanism of the plants against metal stress was thus observed with the increased production of peroxidase, and the reduced growth by the decrease in the chlorophyll content.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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