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# Selenium Mediated Stress Amelioration of Chromium in *Brassica juncea* L. Plants

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**Abstract :** In the present work, stress ameliorative properties of Selenium (Se) were studied in *Brassica juncea* L. plants subjected to Chromium (Cr) stress. *B. juncea* plants were raised in earthen pots and were treated with different concentration and combination of Se and Cr. The plants were harvested after 60 days and were analysed for morphology, antioxidants (glutathione, ascorbic acid, tocopherol), pigments (chlorophylls a, b, carotenoids, xanthophylls), phenols, flavonoids, glycine betaine, proline and MDA contents. The results suggested that Se treatments proved to enhance the tolerance of the plants to Cr stress.

**Key Words: Abiotic stress, Heavy metals, *Brassica juncea*.**

## INTRODUCTION

Heavy metals are one of the most hazardous contaminants that pose a serious threat to plant and animal biosystems. Discharge of heavy metals in high quantities is mainly due human activities like mining, smelting, municipal wastes, sewage, fertilizers, pesticides, fossil fuel burning, etc. These metals usually enter the food chain *via* roots of plants and then reach to upper trophic levels. Many heavy metals like iron (Fe), magnesium (Mg), copper (Cu), zinc (Zn), etc. are important micronutrients. However, their concentrations, when exceed the permissible amounts prove harmful for plants. There are certain metals which are non-essential and become toxic even at trace amounts. One of such metals is chromium (Cr) which has a number of industrial applications which leads to its increasing contamination in the environment. The most stable oxidation states of this transition element are Cr (III) and Cr (VI) and latter is reported to be the most toxic to both plants and animals[1, 2]. It increases the production of reactive oxygen species (ROS) thereby causing oxidative stress in plant system and also interferes with the metabolic processes leading to low germination, stunted growth, reduced photosynthesis, etc.[3]. Plants possess antioxidative defence system which aids in scavenging of these ROS thereby maintaining a balance between their production and amelioration and therefore protects them from oxidative damage.

Selenium (Se) is one of the most widely distributed elements in the Earth's crust, however, its distribution is quite uneven[4]. It is a non metal that resembles sulphur (S) in its physical and biochemical properties[5]. Due to this similarity, it competes with S in its uptake and metabolism. Though, Se has not yet been considered as an essential micronutrient, but many workers have reported its beneficial effects at lower concentrations[6-8]. Its role in fortifying the defence system of stressed plants and improvement in tolerance to stress has been established in many reports.

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*Brassica juncea* is an oil yielding edible crop and is a known hyperaccumulator for heavy metals. It has high biomass and its property to accumulate heavy metals indicates that it has a strong defence mechanism which makes it an ideal plant to study the responses of stress and ameliorative properties of Se. In the present study, *Brassica juncea* plant were exposed to Cr stress and stress alleviation properties of Se were studied by analysing the morphology, antioxidants, pigment system, phenols, flavonoids, glycine betaine, proline and MDA contents.

## MATERIALS AND METHODS

### Raising of Study Material

*Brassica juncea* L. (RLC 1) was used as the study material and the seeds were procured from Punjab Agricultural University, Ludhiana. The surface sterilized seeds were dipped in distilled water for two hours. These were then sown in earthen pots containing a mixture of soil, sand and manure in the ratio of 3:1:1. These pots were of uniform size and contained 5 Kg of the soil mixture. Before sowing the seeds, the treatment solutions comprising of 2, 4 and 6 µM Se ($Na_2SeO_4$) and 300 µM Cr ($K_2CrO_4$) alone and in combination, were added to the pots. The plants were allowed to grow under natural conditions and they were harvested after 60 days. The experiment was conducted in triplicates under same conditions.

### Growth Parameters

The shoots and roots of 60 days old plants were separated and their lengths were measured.

### Biochemical Analysis

Following biochemical parameters were analysed:

1. Lipid Peroxidation

Lipid peroxidation was measured by determining the content of malondialdehyde (MDA) by the method of Heath and Packer[9]. Shoot tissue (1 g) was homogenised in 3 ml 0.1% trichloroacetic acid (TCA) and then mixed with equal volume thiobarbituric acid (TBA) solution (0.5% TBA in 20% TCA) heated to 95 ºC for 30 minutes. The reaction mixture was then quickly cooled in an ice bath and the absorbance of the supernatant was recorded at 532 and 600 nm. The calculations were carried out using 155 $mM^{-1}cm^{-1}$ as extinction coefficient.

2. Non Enzymatic Antioxidants

*a. Glutathione Content*

The content of glutathione (GSH) was measured by the method given by Sedlak and Lindsay[10]. Plant extract was prepared in Tris buffer (0.2 M, pH-8.2). The reaction mixture contained 100 µl of extract, 1 ml of Tris buffer, 50 µl of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and 4 ml of absolute methanol. It was incubated at room temperature for 15 minutes and then centrifuged at 3000xg for 15 minutes. The absorbance of the supernatant obtained was read at 412 nm. The calculation was carried out by using GSH as a standard.

*b. Ascorbic Acid*

Estimation of ascorbic acid was done by method given by Roe and Kuether[11]. The plant extract was prepared in Tris buffer (50 mM, pH-10) under ice cold conditions. The reaction was started with 0.5 ml of extract, 100 mg activated charcoal, 4 ml distilled water and 0.5 ml of 50% TCA. It was mixed well then and filtered with Whatman filter paper No.1 and 0.4 ml of DNPH reagent was added to 1 ml of filterate. The reaction mixture was incubated for 3 hours at 37 ºC followed by cooling in ice bath and addition of cold $H_2SO_4$ (65%). The absorbance was read at 520 nm. The content was calculated by using standard ascorbic acid.

*c. Tocopherol*

Tocopherol estimation was done by the method of Martinek[12]. Plant extract obtained after homogenization of shoot tissue in 3 ml of Tris buffer (50 mM, pH-10) was mixed with 0.12% $FeCl_3.6H_2O$ in absolute ethanol and distilled water (0.5 ml each) and shaken vigorously to get precipitates. Xylene (0.5 ml) was added and vortexed for 30 seconds followed by centrifugation at 3000 x g for 10 minutes. Top layer of xylene (0.5 ml) was mixed with equal volume of 0.12% of 2,4,6-tripyridyl-S-triazine (TPTZ) prepared in n-propanol. The absorbance was measured at 600 nm and calculation was made from standard curve obtained from standard tocopherol.

**3. Pigments**

*a. Chlorophyll and Carotenoids*

The method of Arnon[13] was used to assay the pigments. One gram of shoots was homogenized in 4 ml of 80% acetone under ice cold conditions. The extracts were centrifuged at 10,000 x g for 15 minutes. The supernatant was used to measure chlorophyll a, b and

total chlorophyll spectrophotometrically at the wavelength of 645 and 663 nm and carotenoids were calculated by taking absorbance at 483 and 510 nm.

*b. Xanthophylls*

Estimation of xanthophylls was carried out by method given by Lawrence[14]. The dried plant sample (0.05 g) was extracted with an extractant comprising of hexane, acetone, absolute alcohol and toluene (10:7:6:7). To the extract, 2 ml of 40% methanolic KOH followed by heating at 56 ºC for 15 minutes. The reaction mixture was incubated in dark for 1 hour. To this 30 ml of hexane was added and the volume was made 100 ml by adding 10% $Na_2SO_4$. It was again incubated in dark for 1 hour. The upper phase of the reaction mixture was collected and volume was made to 50 ml by adding hexane. The calculations were made by measuring the absorbance at 474 nm on spectrophotometer.

4. Proline

Proline content was estimated by method given by Bates *et al*[15]. *B. juncea* shoots were in 10 ml of 3% sulphosalicylic acid and centrifuged at 13,000 x g for 10 minutes. To the supernatant, equal volumes of acid ninhydrin and glacial acetic acid was added and boiled for 1 hour at 100 ºC. The test tubes were then kept in ice bath in order to terminate the reaction. To these, 4 ml of toluene was added and was shaken vigorously for 30 seconds. The toluene layer was then allowed to separate from the aqueous phase. The absorbance of toluene layer was recorded at 520 nm and proline content was determined by plotting a standard curve of L-proline.

5. Total Phenols

Method of Singleton and Rossi[16] was used to estimate the content of total phenols in the plant samples. The air dried plant material (0.400 g) was mixed with 40 ml of 60% ethanol and heated at 60 ºC for 10 minutes. The extract was filtered and the final volume was made upto 100 ml with 60% ethanol. The stock (2.5 ml) diluted to 25 ml with distilled water and 2 ml of it was mixed with 10 ml of FC reagent (diluted 10 times). After 5 minutes, 8 ml of 7.5% of $Na_2CO_3$ was added. The reaction mixture was incubated for 2 hours and absorbance was read at 765 nm. The content of total phenol was calculated from a standard curve prepared by running gallic acid as a standard.

6. Flavonoids

Flavonoid content was estimated by a protocol proposed by Zhishen *et al*[17]. Plant sample (100 mg) was extracted with 3 ml of absolute methanol and then filtered with Whatman No. 1 filter paper. To 1 ml of plant extract, 4 ml of double distilled water was added and then 0.3 ml each of 5% $NaNO_2$ and 10% $AlCl_3$ solutions were added. The reaction mixture was given incubation for 5 minutes and 2 ml of 4% NaOH was added followed by addition of 2.4 ml of distilled water. Pink colour developed on addition of NaOH. The absorbance was read at 510 nm and standard curve was made using rutin.

7. Glycine Betaine

Estimation of glycine betaine content was carried out by the method of Grieve and Grattan[18]. The dried plant material (0.5 g) was extracted with 5 ml of toluene-water mixture. The reaction was started by adding 1 ml of 2N HCl and 0.1 ml of $KI_3$ solution to 0.5 ml of extract followed by shaking in ice bath for 90 minutes and addition of ice cold water (2 ml). 1,2-dichloroethane (10 ml) chilled at -10 ºC was poured in the reaction mixture. A stream of fresh air was added for 2 minutes. The two layers separate and absorbance of lower organic layer was measured at 365 nm. The content of glycine betaine was calculated from the standard curve obtained from betaine hydrochloride.

**Statistical Analysis**

The results were statistically analysed by using one-way analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference) test. The values are presented in the form of means ± standard errors (S.E) of the means and the results were considered significant at p d" 0.05.

## RESULTS AND DISCUSSION

It has already been established that exposure of plants to heavy metals induces enhanced ROS generation which further leads to oxidative stress. Cr is one such toxic element which has many deleterious effects on both morphology and physiology of the plant system. Se, however, has been regarded as an essential microelement for some plants which aids in alleviating oxidative stress. In the present study, an effort has been made to have a deeper understanding about the response of morphology, antioxidants, pigment system and some secondary

metabolites in *B. juncea* to Cr and how Se assists in strengthening its resistance against stress.

Cr treatment (300 µM) showed a significant decrease in root and shoot lengths of *B. juncea* plants (Table 1). Among the two, root length decreased most drastically. Similar results were observed in wheat[19, 20], *Vigno mungo*[21] and maize[22]. The reason of decrease root length might be due to the accumulation of Cr in vacuoles of roots and due to this there might be inhibition of either cell division or root cell elongation[23]. Shoot length of plants showed a marked decrease and the reason for this might be the reduced movement of water and nutrients due to reduced root lengths[2]. Application of Se to the growth medium in combination with Cr resulted in enhanced root and shoot lengths (Table 1) thereby signifying the protective effect Se has on plants as a trace element.

Stress leads to excessive production of ROS which are highly reactive and disturb the normal cellular metabolism[24]. ROS generation leads to membrane destabilization which further causes lipid peroxidation. Extent of lipid peroxidation is measured by estimating the content of MDA. Cr treatment caused enhanced MDA content suggesting its toxic effect on *B. juncea* (Table 1). Similar observations were also reported in *B. juncea*[25], Maize[22, 29]. Se addition, however, helped in reduction in MDA content indicating a lower degree of membrane damage and alleviating the damage caused by oxidative stress (Table 1). These results are in conformity with previous reports on cucumber[27], wheat[28] and *B. napus*[29].

Non enzymatic antioxidants play a significant role in scavenging of ROS and augmenting the defence system of the plants. Gluthathione is involved in maintaining the ascorbic acid content in ascorbate-glutathione cycle[30], however, ascorbic acid acts as a reductant and maintains the activity of antioxidative enzymes[31]. Tocopherol, on the other hand, enhances tolerance of the plants by scavenging of singlet oxygen species. In the present study, it was observed that Cr treatment to *B. juncea* decreased the contents of glutathione, ascorbic acid and tocopherol (Table 2). Glutathione content was also found to decrease in reaction to heavy metal stress in *B. napus*[29]. The same report also showed a decrease in ascorbic acid content. A decrease in the content of tocopherol was also observed in broccoli by Pedrero *et al*[32]. When Se was supplied along with Cr, enhancement in the contents of these antioxidants was observed to a significant level leading to the enhanced defence system of the plant (Table 2). Such an increase was also observed in *B. napus*[29, 33] which confirms the involvement of Se in tolerance against stress.

A significant reduction in chlorophylls and carotenoids was observed when *B. juncea* plants were treated with Cr (Table 3). This decrease may be due to the inhibition of biosynthesis of chlorophyll pigments. Vajpayee *et al.*[34] suggested that Cr toxicity can lead to degradation of ä-aminolevulinic acid which is an important enzyme involved in chlorophyll biosynthesis. Reports on plants like *Oryza sativa*[35] and maize[26] showed similarity with our results. Application of Se with Cr led to increase of pigment contents thereby suggesting the restoration of normal functioning of the plants (Table 3). On the other hand, pigment like xanthophylls showed enhanced contents with the induction of stress to the plants (Table 3). Xanthophylls have been reported to enhance during heat and light stresses in rice[36] and thereby enhance the resistance of plant against the stresses.

Proline is an amino acid which aids in free radical scavenging, stabilizes membranes in stress, protects enzymes present in cytoplasm and is one of the major osmoticums of the cytoplasm[37]. In the present study, the content of proline showed an increase when the plants were treated only with Cr (Table 4). Similar results were reported in *Glycine max*[38], *Vigna radiata*[37] and *Camelia sinensis*[39]. On Se supplementation to the stressed plants, the content of proline was found to enhance further which indicated an upsurge in tolerance of plants towards stress (Table 4). Earlier reports documented on cucumber[27] and wheat[28] showed similar results.

An increase in content of total phenols was observed in the present study upon treatment of *B. juncea* plants with Cr (Table 4). This might be a protective effect of these compounds which are thought to alleviate metal stress by chelation of heavy metals and scavenging of ROS[40, 41]. Upon treatment with Se, the content was further found to increase (Table 4) which suggests that Se aids in fortifying the defence system of the plants. This result is in conformity with previous studies carried on wheat plants where Se treatment significantly enhanced phenols in UV-B stressed plants[28].

Flavonoids are a group of secondary metabolites which are involved in diverse biological functions and plant protection against various stresses[42]. The stress protective function of flavonoids against heavy metals might be due to their ability to form complexes which further enhance the plant's capacity to tolerate metal stress[40, 43-46]. The present study shows an increase in flavonoid content when *B. juncea* plants were treated with Cr (Table 4). An increase in flavonoid content in response to heavy metal was also reported in *Ginkgo biloba*[47] and *Ononis arvensis*[48]. When the stressed plants were treated with different Se concentrations, it was found that the content increased further (Table 4). Similar results were reported in UV-B stressed wheat seedlings where Se enhanced the stress tolerance by aiding in accumulation of flavonoids[28].

Glycine betaine is an osmoprotectant which has an important role in stress. In the present piece of work, an increase was observed in the content of glycine betaine upon induction of Cr stress to plants (Table 4). Many studies have also reported an increase in its accumulation in response to various stresses like drought[49], salt[50], cold[51] and heavy metal[52, 53]. Upon treatment with Se, the content of glycine betaine was further found to enhance (Table 4). Glycine betaine accumulation has been reported to enhance tolerance of plants by protecting RuBisCO enzyme[54], photosystem II[55] and maintenance of the turgor pressure[56]. Hence, Se might have an effect on glycine betaine content which further leads to plant protection against stress.

**Table 1. Effect of different concentrations and combinations of Se and Cr on Shoot Length, Root Length and Malondialdehyde content of *B. juncea* plants.**

| Treatments (µM) | Shoot Length (cm) Mean ± S.E | Root Length (cm) Mean ± S.E | Malondialdehyde (mMol/g F.W) Mean ± S.E |
|---|---|---|---|
| Control | 16.367 ± 0.392 | 8.233 ± 0.667 | 4.628 ± 0.031 |
| Se-2 | 15.9 ± 0.579 | 7.133 ± 0.28 | 4.567 ± 0.0267 |
| Se-4 | 17.067 ± 0.982 | 7.68 ± 0.475 | 4.12 ± 0.045 |
| Se-6 | 17.32 ± 0.808 | 7.38 ± 0.578 | 5.866 ± 0.037 |
| Cr-300 | 14.22 ± 0.328 | 5.117 ± 0.116 | 6.034 ± 0.076 |
| Cr+Se-2 | 15.32 ± 0.641 | 7.5 ± 0.482 | 4.941 ± 0.0195 |
| Cr+Se-4 | 17.62 ± 0.209 | 5.667 ± 0.502 | 4.606 ± 0.0267 |
| Cr+Se-6 | 17.3 ± 0.161 | 6.75 ± 0.548 | 5.677 ± 0.032 |
| | $F_{(df=7,16)}$ 4.1399*; HSD = 2.844 | $F_{(df=7,16)}$ 4.724*; HSD = 2.373 | $F_{(df=7,16)}$ 308.306*; HSD = 0.1977 |

Data shown are Mean ± SE of three experiments. *Significant at pd"0.05.

**Table 2. Effect of different concentrations and combinations of Se and Cr on Glutathione, Ascorbic Acid and Tocopherol contents of *B. juncea* plants.**

| Treatments (µM) | Glutathione (mg/g F.W) Mean ± S.E | Ascorbic Acid (mg/g F.W) Mean ± S.E | Tocopherol (mg/g F.W) Mean ± S.E |
|---|---|---|---|
| Control | 1.555 ± 0.062 | 0.0487 ± 0.0015 | 4.198 ± 0.415 |
| Se-2 | 1.412 ± 0.066 | 0.0504 ± 0.0028 | 4.283 ± 0.194 |
| Se-4 | 1.317 ± 0.114 | 0.0499 ± 0.0017 | 4.565 ± 0.0822 |
| Se-6 | 0.963 ± 0.49 | 0.0645 ± 0.0051 | 4.112 ± 0.175 |
| Cr-300 | 0.997 ± 0.027 | 0.036 ± 0.0004 | 3.986 ± 0.084 |
| Cr+Se-2 | 0.879 ± 0.0359 | 0.0417 ± 0.0004 | 9.493 ± 0.115 |
| Cr+Se-4 | 1.081 ± 0.0296 | 0.0417 ± 0.0003 | 6.654 ± 0.236 |
| Cr+Se-6 | 0.502 ± 0.013 | 0.0354 ± 0.0017 | 4.122 ± 0.126 |
| | $F_{(df=7,16)}$ 64.315*; HSD = 0.2049 | $F_{(df=7,16)}$ 16.359*; HSD = 0.0115 | $F_{(df=7,16)}$ 89.375*; HSD = 1.0104 |

**Table 3. Effect of different concentrations and combinations of Se and Cr on Pigment System of *B. juncea* plants.**

| Treatments (μM) | Total Chlorophyll (mg/g F.W) Mean ± S.E | Chlorophyll a (mg/g F.W) Mean ± S.E | Chlorophyll b (mg/g F.W) Mean ± S.E | Carotenoids (mg/g F.W) Mean ± S.E | Xanthophylls (mg/g D.W) Mean ± S.E |
|---|---|---|---|---|---|
| Control | 0.0925 ± 0.0021 | 0.0558 ± 0.0007 | 0.0364 ± 0.0015 | 0.0336 ± 0.0006 | 8.33 ± 0.53 |
| Se-2 | 0.2096 ± 0.0021 | 0.1569 ± 0.0014 | 0.0509 ± 0.0006 | 0.0535 ± 0.0005 | 3.013 ± 0.169 |
| Se-4 | 0.1195 ± 0.0005 | 0.0789 ± 0.0003 | 0.0401 ± 0.0002 | 0.0338 ± 0.0002 | 3.861 ± 0.308 |
| Se-6 | 0.1392 ± 0.00048 | 0.0931 ± 0.0006 | 0.0387 ± 0.0002 | 0.0416 ± 0.0002 | 3.484 ± 0.124 |
| Cr-300 | 0.0842 ± 0.0013 | 0.0551 ± 0.0008 | 0.0286 ± 0.0004 | 0.0313 ± 0.0002 | 17.091 ± 0.454 |
| Cr+Se-2 | 0.177 ± 0.00035 | 0.1212 ± 0.0002 | 0.0554 ± 0.0002 | 0.0477 ± 0.00006 | 3.531 ± 0.082 |
| Cr+Se-4 | 0.0845 ± 0.00083 | 0.0531 ± 0.0004 | 0.0311 ± 0.0004 | 0.033 ± 0.0002 | 2.589 ± 0.124 |
| Cr+Se-6 | 0.0688 ± 0.0013 | 0.0442 ± 0.0008 | 0.0243 ± 0.0005 | 0.0299 ± 0.0003 | 6.779 ± 0.215 |
| | $F_{(df=7,16)}$ 1489.02*; HSD = 0.00633 | $F_{(df=7,16)}$ 2938.637*; HSD = 0.00358 | $F_{(df=7,16)}$ 273.796*; HSD = 0.00317 | $F_{(df=7,16)}$ 651.335*; HSD = 0.00164 | $F_{(df=7,16)}$ 271.789*; HSD = 1.449 |

Data shown are Mean ± SE of three experiments. *Significant at pd"0.05.

**Table 4. Effect of different concentrations and combinations of Se and Cr on Proline, Total Phenols, Flavonoids, Glycine Betaine of *B. juncea* plants.**

| Treatments (μM) | Proline (μg/g F.W) Mean ± S.E | Phenols (μg/mg D.W) Mean ± S.E | Flavonoids (μg/mg D.W) Mean ± S.E | Glycine Betaine (μg/mg D.W) Mean ± S.E |
|---|---|---|---|---|
| Control | 202.17 ± 1.92 | 10.92 ± 0.263 | 4.16 ± 0.087 | 1.276 ± 0.032 |
| Se-2 | 210.16 ± 1.21 | 18.52 ± 0.137 | 3.99 ± 0.065 | 1.869 ± 0.035 |
| Se-4 | 219.52 ± 0.86 | 14.19 ± 0.093 | 3.71 ± 0.107 | 2.209 ± 0.076 |
| Se-6 | 228.65 ± 2.06 | 18.23 ± 0.056 | 3.77 ± 0.071 | 2.402 ± 0.035 |
| Cr-300 | 299.56 ± 2.06 | 19.73 ± 0.032 | 5.62 ± 0.105 | 2.696 ± 0.034 |
| Cr+Se-2 | 309.13 ± 1.39 | 19.01 ± 0.13 | 5.87 ± 0.037 | 1.725 ± 0.100 |
| Cr+Se-4 | 319.86 ± 1.81 | 18.79 ± 0.244 | 5.95 ± 0.059 | 2.5 ± 0.048 |
| Cr+Se-6 | 282.53 ± 1.2 | 24.93 ± 0.195 | 5.63 ± 0.065 | 2.345 ± 0.023 |
| | $F_{(df=7,16)}$ 897.856*; HSD = 7.94 | $F_{(df=7,16)}$ 625.099*; HSD = 0.803 | $F_{(df=7,16)}$ 168.781*; HSD = 0.381 | $F_{(df=7,16)}$ 77.433*; HSD = 0.264 |

Data shown are Mean ± SE of three experiments. *Significant at pd"0.05.

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