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Selenium ameliorates chromium toxicity through modifications in pigment system, antioxidative capacity, osmotic system, and metal chelators in *Brassica juncea* seedlings

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ABSTRACT

The present study was designed to understand the protective role of Selenium (Se) in *Brassica juncea* seedlings cultivated under chromium (Cr) stress. The seedlings were raised in Petri dishes containing solutions of Cr (300 μM) and Se (2, 4 and 6 μM) in unary and binary combinations. The 15 days old seedlings were harvested and assessed for pigments, stomatal morphology and stomatal density, total antioxidative capacity, osmolytes and metal chelators. The results showed that the seedlings treated with Cr showed a significant decrease in the contents of chlorophyll (40.95%) and carotenoids (21.38%). Cr application also caused reduction in stomatal density (42.10%) and negatively affected the morphology of stomata. Se supplementation reduced the toxicity of Cr by increasing the pigment contents and improving the morphology of leaves. Relative gene expression of chlorophyllase reduced, while that of phytoene synthase and chalcone synthase enhanced with Se application which supported the above observations. Cr stress increased the levels of lipid- and water-soluble antioxidants by 68.25 and 32.35%, respectively; however, supplementation of Se further increased their contents. Proline, glycine betaine, trehalose, and osmolality increased by 155.19, 49.18, 83.52, and 120.92%, respectively, in seedlings subjected to Cr stress. Further increment in the above parameters was observed by the application of Se to Cr-stressed seedlings. Seedlings treated with 300 μM Cr showed an increase of 27.27% in the content of total thiols, 127.27% in the content of non-protein thiols, and 21.05% in that of protein-bound thiols, and supplementation of Se showed additional an increase by 28.57, 34.00, and 26.08%, respectively, over those in the Cr-treated seedlings. In conclusion, Cr hampered the normal functioning of the *B. juncea* seedlings and Se application mitigated the negative impact through modulation in the contents of various osmolytes, antioxidants, and other metabolites. Thus, Se application might help the seedlings to withstand stress by strengthening their antioxidant system, osmoregulation, and metal-chelating ability.

1. Introduction

The concentration of heavy metals in urban and agricultural soils is on a continuous rise due to various natural and anthropogenic reasons. Mining and many industrial processes lead to heavy metal contamination of the soils. Industrial effluents also contain heavy metal and their oxide as nanoparticles which have toxic impacts on the plants (Rastogi et al. 2017). They enter the plant systems and ultimately produce hazardous effects on the entire food chain (Handa et al. 2018). Chromium (Cr) is one of those heavy metals, which is non-essential for the living organisms and therefore, even at trace amounts, it can produce acute effects (Handa et al. 2017, 2018). Because of its immense applications in industries, it is widely released as an industrial pollutant. Two of the most stable forms of Cr are Cr(III) and Cr(VI) with the latter being more toxic to plants (Panda and Patra 2000; Han et al. 2004). This toxicity of Cr(VI) is because of its presence in the form of oxy anions (chromate or dichromate), whereas Cr(III) usually occurs in the form of sulfates, hydroxides, and oxides or is bound to organic compounds of soil and water (Zayed and Terry 2003). Presence of Cr(VI) in soil and water affects the uptake of nutrients because of its interaction with the nutrients (Zupančič et al. 2004). In plants, the toxic effects of Cr are visible on seed germination as well as on growth and biomass production (Singh et al. 2013). A number of studies have reported such effects in *Oryza sativa*, *Glycine max*, and *Triticum aestivum* (Amin et al. 2014; Nagarajan and Ganesh 2014; Ghani et al. 2015). Cr toxicity also interferes with important biomolecules leading to changes in

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major metabolic pathways in plants. The loss in the content of photosynthetic pigments because of Cr has been reported in plants such as *Hibiscus esculentus*, *Catharanthus roseus*, and *Corchorus olitorius* (Amin et al. 2013; Islamit et al. 2014; Rai et al. 2014). Exposure of plants to Cr also leads to enhanced production of reactive oxygen species (ROS), which results in altered ROS metabolism (Singh et al. 2013). Various enzymatic and non-enzymatic antioxidants in plants, whether lipid- or water-soluble, play significant roles in scavenging of ROS. It has been established in studies on plants such as *T. aestivum*, *Solanum nigrum, Cyamopsis tetragonoloba* that Cr stress also induces the osmotic system, which aids in osmoregulation by the release of compatible solutes (Datta et al. 2011; Sangwan et al. 2013; Teixeira et al. 2013).

Selenium (Se), the sister element of sulfur, has become a vital candidate and has gained the status of an essential micronutrient because of its important role in normal plant growth and development (Pilon-Smits and Quinn 2010). The biological activity of Se is attributed to its similarity with sulfur, which allows its uptake and incorporation in important biomolecules (Pilon-Smits and Quinn 2010). The most stable forms of Se are selenate [Se(VI)] and selenite [Se(IV)], which exist abundantly in the environment (Sharma et al. 2011). Of the two forms of Se, the bioavailability of Se(VI) is higher, and hence, it is readily taken up by active processes (Terry et al. 2000; White et al. 2004). Further, Se(VI) is not accumulated in the roots of plants and is translocated to the shoots and eventually to the chloroplasts via the sulfur assimilation pathway (Läuchli 1993; Terry et al. 2000). This leads to the formation of selenocompounds, which have great biological significance. However, stress ameliorative properties against heavy metals by many compounds have been reported in earlier studies (Mohanty and Patra 2011; Choudhary et al. 2012; Song et al. 2012, 2014), investigation on the role of Se as a stress ameliorator for various abiotic stresses is an area of active research (Ahmad et al. 2016). Its exogenous application at appropriate levels has been documented to provide resistance against several stresses, including drought, temperature, UV-B, and heavy metal (Hasanuzzaman and Fujita 2011; Pukacka et al. 2011; Yao et al. 2011; Akladious 2012; Ahmad et al. 2016). Many members of the family Brassicaceae such as *Brassica napus*, *Brassica oleracea* and *B. juncea* are considered to be the primary accumulators of Se (Hasanuzzaman et al. 2012). Also, *B. juncea* can accumulate various heavy metals including Cr in its above ground parts (Sytar et al., 2015). Therefore, in the present study, *B.juncea*, was used as a model plant to understand the role of Se against Cr stress. The hypothesis thus tested was the ameliorative role of Se by assessing photosynthetic system, antioxidant and osmotic machinery, and metal chelating ability of *B. juncea* plants.

2. Materials and methods

2.1. Plant material and experimental setup

The seeds of *B. juncea* (var. RLC 1) were sterilized with 0.01% mercuric chloride ($HgCl_2$), soaked in distilled water for 2 h (h), and germinated in Petri dishes lined with Whatman No. 1 filter paper moistened with 3 ml solutions of sodium selenate (Na_2SeO_4) (2, 4, and 6 μM) and potassium chromate (K_2CrO_4) (300 μM), in unary and binary treatments. The concentrations of the elements were decided on the basis of preliminary experiments by obtaining the most stimulatory concentration of Se and 50% inhibitory concentration (IC50) of Cr. The treatment solutions were prepared in half strength Hoagland's nutrient medium and the experiment was conducted in triplicates with 16 h photoperiod and 25 ± 2 °C temperature. The seedlings were harvested after 15 days for the estimation of various parameters.

2.2. Plant pigments

The method of Arnon (1949) was used for the estimation of total chlorophyll and carotenoids. The homogenates of seedlings were prepared in 80% acetone, and these were centrifuged at 13,000 × *g* for 20 min at 4 °C. For estimation of the chlorophyll content, the absorbance of the supernatant was recorded at 645 and 663 nm, whereas for carotenoids, the absorbance was recorded at 480 and 510 nm in the UV-visible range using a spectrophotometer (UV-Visible PC Based Double Beam Spectrophotometer, Systronics 2202).

The content of anthocyanins was determined following the protocol of Mancinelli (1984). The homogenates of seedlings were extracted using a mixture of methanol, distilled water, and hydrochloric acid (HCl) in the ratio 79:20:1. The supernatants were collected after centrifugation, and the absorbance was spectrophotometrically measured at 530 and 657 nm.

The xanthophyll content was determined following the method of Lawrence (1990). Dry and powdered seedling samples were immersed in an extraction mixture (30 ml) consisting of hexane, acetone, absolute alcohol, and toluene (10:7:6:7) and were shaken for 10–15 min (min). This was followed by the addition of 2 ml of 40% methanolic potassium hydroxide and vigorous shaking. The mixture was heated at 56 °C in a water bath and incubated for 1 h in the dark at room temperature. An additional 30 ml of hexane was added, and the reaction mixture was shaken well. Thereafter, the volume was made upto 100 ml by the addition of 10% sodium sulfite (Na_2SO_3), and the mixture was vigorously shaken for 1 min. The reaction mixture was incubated for another 1 h, and the two phases were allowed to separate. After the incubation, the upper phase was removed, and its volume was made upto 50 ml by adding hexane. The absorbance of this phase was spectrophotometrically determined at 474 nm.

2.3. Stomatal morphology and stomatal density

Scanning electron microscope (SEM) (EvoLS 10, Carl Zeiss) was used to study the morphology and density of stomata on the lower surface of cotyledonary leaves of 15-days-old *B. juncea* seedlings. The density was calculated by determining the number of stomata mm^{-2} of leaf area.

2.4. Total antioxidative capacity

The antioxidative capacities of lipid- and water-soluble antioxidants in *B. juncea* seedlings were determined using an antioxidant analyzer (PHOTOCHEM BU, Analytik Jena). For the determination of lipid-soluble antioxidants, the seedling samples were prepared in absolute methanol by homogenization and centrifugation under ice-cold conditions. An aliquot of 10 μl of supernatant was taken and diluted with 490 μl of methanol. For water-soluble antioxidants, the seedling samples were prepared in 50 mM Tris buffer (pH 10) under similar conditions, and dilutions were made as in the case of lipid-soluble antioxidants. Both lipid- and water-soluble antioxidants were estimated using standard reagent kits provided by Analytik Jena, in which free radicals are generated and then scavenged by the antioxidants present in the plant extract. The instrument uses photochemiluminescence method to detect the decrease in the fluorescence intensity. The calibration curves obtained using standards were used to quantify the antioxidative capacities.

2.5. Free proline

The estimation of free proline was done according to the method of Bates et al. (1973). The seedling samples were homogenized in 3% sulfosalicylic acid and centrifuged at 13,000 × *g* for 20 min. Equal volumes of supernatant and acid ninhydrin (1.56 g of ninhydrin in 37.5 ml glacial acetic acid and 25 ml of 6 M *ortho*-phosphoric acid) were taken and boiled in a water bath for 1 h. The reaction mixture was cooled immediately to terminate the reaction. This was followed by addition of toluene and vortexing of the mixture. The mixture was kept at room temperature, and the absorbance of toluene layer was recorded at 520 nm spectrophotometrically.