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Amendment in phosphorus levels moderate the chromium toxicity in *Raphanus sativus* L. as assayed by antioxidant enzymes activities

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ABSTRACT

Chromium ($Z=24$), a d-block element, is a potent carcinogen, whereas phosphorus is an essential and limiting nutrient for the plant growth and development. This study undertakes the role of phosphorus in moderating the chromium toxicity in *Raphanus sativus* L., as both of them compete with each other during the uptake process. Two-factor complete randomized experiment (5 chromium × 5 phosphorus concentrations) was conducted for twenty eight days in green house. The individuals of *R. sativus* were grown in pots supplied with all essential nutrients. The toxic effects of chromium and the moderation of toxicity due to phosphorus amendment were determined as accumulation of chromium, nitrogen, phosphorus in root tissues and their effects were also examined in the changes in biomass, chlorophyll and antioxidant enzyme levels. Cr and N accumulation were almost doubled at the highest concentration of Cr supply, without any P amendment, whereas at the highest P concentration (125 mM), the accumulation was reduced to almost half. A significant reduction in toxic effects of Cr was determined as there was three-fold increase in total chlorophyll and biomass at the highest P amendment. Antioxidant enzymes like superoxide dismutase, catalase, peroxidase and lipid peroxidation were analyzed at various levels of Cr each amended with five levels of P. It was observed that at highest level of P amendment, the reduction percentage in toxicity was 33, 44, 39 and 44, correspondingly. Conclusively, the phosphorus amendment moderates the toxicity caused by the supplied chromium in *R. sativus*. This finding can be utilized to develop a novel technology for the amelioration of chromium stressed fields.

1. Introduction

Chromium ($Z=24$) is a well known toxic heavy metal. The health hazards caused by the Cr exposure range from dermatitis, dermatosis and different types of cancer. The sources of Cr contamination of soils are different industrial operations, smelting, tanning, electroplating etc. The risk of Cr transfer in the food chain from soil is immense, by the process of uptake and accumulation by crop plants (Dube et al., 2003). Cr exists in both mobile and chelated forms. Out of the two stable forms Cr (III) and Cr (VI), former occurs in the bound form with organic matter of soil, whereas the latter exists in the inorganic form, mainly associated with oxygen as chromate and dichromate ions (Shanker et al., 2005).

Adsorption, absorption, accumulation and transformation are the processes by which plants detoxify the metal/metalloid contaminated sites. Above processes come under the umbrella of one phenomenon named as phytoremediation. It is defined by Cunningham and Berti (1993) as the use of plants to remove pollutants from environment or to render them harmless. It is a green and cost effective technology, which can be utilized in place of other expensive technologies. In the process, one or a group of hyperaccumulator species grown as a unit called as constructed ecosystem is being used in developed countries (Azaizeh et al., 2006). Many papers and reviews are written on it (Salt et al., 1998; Schnoor et al., 1995; Shardendu et al., 2003), still, there are many gaps to be filled in. That is, interaction of growth nutrients (e.g. P) with chelated or unchelated metal ions (Dube et al., 2003), e.g. P may interact with Cr in-situ (Wallace et al., 1976; Shanker et al., 2005).

Phosphorus is an essential macronutrient and competes with Cr for uptake (Wallace et al., 1976; Cervantes et al., 2001). Phosphorus is taken up by plants in the form of phosphate ions present as dissolved inorganic phosphorus (DIP) in the water

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; DHAR, dehydroascorbate reductase; DIP, dissolved inorganic phosphorus; DOP, dissolved organic phosphorus; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; GR, glutathione reductase; MDA, malondialdehyde; MDHAR, Monodehydroascorbate reductase; PIP, Particulate inorganic phosphorus; POD, Peroxidase; POP, Particulate organic phosphorus; ROS, Reactive oxygen species; SE, (Standard error); SOD, Superoxide dismutase; UV–vis, Ultra violet–visible

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column of soil particles. Other forms of phosphorus include particulate inorganic phosphorus (PIP), dissolved organic phosphorus (DOP) and particulate organic phosphorus (POP), which have to undergo biotransformation to DIP for phytoavailability (Welch and Lindell, 1992; Holdford, 1997). In plant body, excess phosphorus accumulates as polyphosphate (Eixler et al., 2006; Powell et al., 2009). These polyphosphates may either be acid soluble or acid insoluble form (Miyachi et al., 1964). Acid soluble polyphosphates are utilized in metabolism and synthesis of deoxyribonucleic acid (DNA) and proteins, whereas acid insoluble form is stored in the biomass and is utilized in the condition of starvation of phosphorus (Kuhl, 1974; Powell et al., 2008).

Chromium toxicity in plants generates reactive oxygen species (ROS) causing oxidative stress and lipid peroxidation (Shanker et al., 2005; Subrahmanyam, 2008) in various tissues. ROS is scavenged by various antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and other thiol regulated enzymes like dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) (Clijsters et al., 1999; Shanker et al., 2004). Levels of these antioxidant enzymes can be used as indicators of heavy metal induced metabolic stress in plants (Shanker et al., 2005).

The present study examines the role of different levels of phosphorus amendments on chromium toxicity in *Rapahnus sativus* L. The advantages of this cosmopolitan dining item are its storage root (tuber) and shorter growth cycle. But surprisingly, it is left uninvestigated so far. The experiment is done with the design in which five different concentrations of Cr has been taken and at each Cr level, there were five amendments of P concentration. The variations in chlorophyll and biomass content, along with root tissue storage of Cr, P and N in *R. sativus* have been determined. Furthermore, to examine the effect of P on Cr induced oxidative-toxicity, the variations in antioxidant enzymes (superoxide dismutase, catalase, peroxidase) and lipid peroxidation levels (as malondialdehyde content) in root tissues have been determined.

2. Materials and methods

2.1. Experimental design and plant growth

Two-factor complete randomly designed experiment (in 5×5 pattern) was set up to examine the effects of amendments of phosphorus on chromium toxicity in *Raphanus sativus* L. Plants were supplied with five concentrations of Cr (viz. 2.0, 3.5, 5.0, 6.5 and 8.0 mM). Each level of Cr was amended with five concentrations of phosphorus (viz. 25, 50, 75, 100 and 125 mM). P was supplied as sodium dihydrogen phosphate salt and Cr (as Cr^{6+}) was supplied as potassium dichromate salt. The controls were, 0.0 mM Cr with 125 mM P and 0 mM P with 2.0–8.0 mM Cr.

Seeds of *R. sativus* were wrapped in sterilized tissue paper towels soaked in half strength nutrient solution (Shardendu et al., 2012), and were allowed to germinate for 72 h. Meanwhile, for the pot experiment, refined sand was prepared. Sand was treated with 3% hydrochloric acid (HCl), for leaching out all the minerals adsorbed on to the sand. The HCl treated sand was then soaked in distilled water for 5 h, and washed three times with doubled distilled water so that the pH of sand rises from acidic to neutral. Later, sand was air dried and autoclaved. Each pot was filled with 4 kg prepared sand and 2 L full strength nutrient solution. The pots without seedlings were transferred to the green house. The seedlings of *R. sativus* were planted at a density five per pots. The seedlings were allowed to grow with natural light and dark conditions where temperature was maintained at 25 ± 5 °C. The volume of nutrient solution was maintained in every three days.

After 10 days of seedling growth, it was thinned to one per pot. Treatments of Cr and P were started with the eleven days old seedlings. The 25 pots were supplied with Cr and P as above mentioned (5×5) fashion along with the controls. Each combination of Cr and P treatment had three replicates, leading to 75 treatment pots.

A similar experiment with 75 pots as above were done with the seedling density of five per pot and the variation in antioxidant enzyme levels were measured on the first day of treatment, at the intervals of 0, 1, 3, 6 and 12 h.

2.2. Determination of dry weight biomass and chlorophyll

Plants were harvested after twenty eight days of treatments of Cr and P and were separated into root and shoot parts. Dry weight biomass of shoots+roots was determined for every treatment. Chlorophyll was measured in the fresh leaves and root parts were utilized for determination of Cr, P and N.

Roots were initially washed under gently running tap water to remove loosely adhered sand particles followed by rinsing with 3% HCl for leaching out of minerals adsorbed on the surface of roots. The shoots and acid rinsed roots were washed at least three times with double-distilled water, and dried in a drought oven at 80 °C till the achievement of constant weight. Dry weight (DW) biomass (in grams) per plant was determined using Electronic Balance (Oriental Sales, India).

Chlorophyll a, b were estimated by its extraction from leaves using 80% acetone followed by determination of OD_{663} and OD_{645} using UV–vis spectrophotometer (*SYSTRONICS* 2202, India) and then calibrating these values in formulae devised by Maclachlan and Zalik (1963).

2.3. Digestion of plant material and determination of Cr, P and N

For analysis of Cr, P and N, dried root samples were grinded using stainless steel grinder (Philips, India) and meshed to achieve size < 200 μm. The ground samples were digested with different procedures. For Cr analysis, 500 mg of ground materials were digested in 2+1 nitric acid: perchloric acid using technique as described by Walinga et al. (1995) with minor modifications. Weighed samples were incubated in the acid mixture overnight to overcome the vigorous reaction phase, followed by placing the conical flask containing reaction mixture in heating block and allowed to boil at 150 °C till the dense white fumes of perchloric acid evolved. The flask was removed and the mixture was allowed to cool. Again 15 mL of acid mixture was added and again heated at the same temperature till the digested plant debris turned pure white. The flask was then removed and the digested product was filtered through Whattman 42 filter paper. The filtrate was adjusted to volume of 50 mL with double-distilled water for further analysis of Cr. Total Cr was estimated as per American Public Health Association (APHA, 2005a) where total Cr has been converted to Cr^{6+} before determination UV–vis spectrophotometrically. Conversion was achieved by treating 5 mL of acidified (with 1+1 sulfuric acid: double distilled water) digest with potassium permanganate and sodium azide. Color was developed by addition of diphenyl carbazide reagent and OD_{540} was recorded using spectrophotometer. Concentration of Cr (in micrograms per gram DW) was determined using standard graph.

For the analysis of P and N, the ground-samples were digested by the method described by Langner and Hendrix (1982) with minor modifications. 25 mg ground-sample was boiled in the 150 mL of (1+1) digestion mixture (9 mg L^{-1} sodium hydroxide+20.1 mg L^{-1} potassium peroxodisulphate): doubled distilled water, at 120 °C for 2 h. The digested product was filtered through Whattman 42 filter paper and the filtrate was used for further determination of N and P. Phosphorus in the digested product was determined by stannous chloride method (APHA, 2005b)). Ammonium molybdate (0.4 mL) and stannous chloride (0.05 mL) were added to 10 mL of acidified digested product and OD_{690} was recorded using spectrophotometer after 10–12 min of incubation. Concentration of phosphorus (in milligrams per gram DW) in the digest was measured using a standard graph.

Total nitrogen in the digested product was determined as nitrate–nitrogen following the phenol–disulphonic acid method (Eastoe and Pollard, 1950). 50 mL of digest was evaporated to dryness on a hot plate (temperature=80 °C) followed by the dissolution of precipitate in 2+20 phenol disulphonic acid: double distilled water. Dropwise ammonia solution was added till the solution turned yellow and volume was made up to 100 mL. OD_{410} was recorded using spectrophotometer and the concentration of N (in milligrams per gram DW) was determined using standard graph.

2.4. Extraction and assay of antioxidant enzymes and lipid peroxidation

One plant from each pot containing five individuals of *R. sativus* was harvested on the first day of treatment at the intervals of 0, 1, 3, 6 and 12 h for the analysis of antioxidant enzymes. For extraction of enzymes, 1 g of frozen roots was homogenized in ice cold 0.1 M Tris–HCl buffer maintained at pH 7.8 along with 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mL of 4% polyvinyl pyrrolidone and 1 mM dithiotreitol, followed by filtration of homogenate and centrifugation at 20,000g at 4 °C for 20 min The supernatant was used for further enzyme assays.

Superoxide dismutase (SOD) [EC 1.15.1.1] activity was determined as per discussed by Minami and Yoshikawa (1979). The reaction mixture consisted of 50 mM Tris–cacodylic buffer (Sodium salt), pH 8.2, 0.1 mM EDTA, 1.4% TritonX-100, 0.055 μM nitroblue tetrazolium (NBT), enzyme extract and 16 μM pyragallol, which initiated the reaction. The reaction was incubated at 37 °C for 5 min. The reaction was terminated by adding a mixture containing 0.35 M formic buffer, pH 3.5, 0.6% Triton-X 100 and 3.5% formaldehyde. The OD_{540} of the reaction product was recorded and the activity was expressed in Units (U) per milligram FW (where 1 U represented the amount of enzyme that caused the inhibition of NBT reduction by 5%). Catalase (CAT) [EC 1.11.1.6] activity was determined by following the method of