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EFFECT OF CR^{+6} AND CHELATING AGENTS ON GROWTH, PIGMENT STATUS, PROLINE CONTENT AND CHROMIUM BIOAVAILABILITY IN RICE SEEDLINGS

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Abstract- Hexavalent Chromium (Cr^{+6}) induced several toxicological changes in the developing rice (*Oryza sativa* L.) seedlings as showed from present *in vitro* hydroponic study. Growth parameters, physiological and biochemical alterations in rice were studied in response to the application of toxic concentration of Cr^{+6} with addition of chelating agents. Rice seedlings growing in nutrient solutions supplemented with Cr^{+6} (100µM) showed noticeable decrease in root and shoot length, fresh and dry matter; whereas addition of chelating agents like EDTA, DTPA and EDDHA with Cr^{+6} at 10 µM stimulates the growth. Chlorophyll and carotenoid biosynthesis was also declined with the treatment of Cr^{+6} (100µM), but application of Cr^{+6}-EDTA (10µM) amplified the pigment biosynthesis. Rice seedlings treated with Cr^{+6}(100µM) showed increased proline content and high Cr accumulation in root whereas treatments of Cr^{+6}-DTPA (10µM) enhanced the shoot Cr bioaccumulation as compared to other treatments. Supplementation of Cr^{+6}-EDTA (10µM) showed increased phyto-extraction of Cr in roots of rice seedlings. Transportation index (Ti) of seedlings grown in Cr^{+6} (10µM) and Cr^{+6}- DTPA (10µM) was found more in comparison to others. The variations in the total accumulation rate (TAR) of Cr of different seedlings were negligible.

Key words: Chelating agents, Chromium bioaccumulation, Hexavalent chromium, *Oryza sativa* L.

Abbreviations: EDTA (Ethylene Di- amine Tetra Acetic acid); DTPA (Di- ethylene Tri-amine Penta Acetic acid); EDDHA (Ethylene Di-amine Di-ortho-Hydroxy Phenyl Acetic acid)

Introduction

Soil contamination is a major environmental concern due to dispersal of industrial and urban wastes generated by human activities. Disposal of wastes, accidental and process spillage, mining and smelting of ores, sewage sludge application to agricultural soils are responsible factors for contamination of our ecosystem. However, elevated concentrations of both essential and nonessential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants and chromium (Cr) is one of them. The metal has two stable forms which exists in nature, those are trivalent (Cr^{+3}) and hexavalent (Cr^{+6}) ones.

Out of these two forms of chromium, Cr^{+6} is found to be toxic and carcinogenic. Several toxic effects of Cr^{+6} have been reported earlier by researchers [12, 26, 16, 15]. These abiotic stresses induced by Cr^{+6} can be observed through the changes in plant morphology and metabolism. Chelates are the chemical substances which render insoluble cations into soluble form. Although several studies have been conducted on the impact of Cr on of growth and metabolism of plants [8, 26, 5], the role of combined effects of Cr^{+6} and chelating agents viz. EDTA (Ethylene Di- amine Tetra Acetic acid), DTPA (Di- ethylene Tri-amine Penta Acetic acid) and EDDHA (Ethylene Di-amine Di-ortho-Hydroxy Phenyl Acetic acid) on phytotoxic effects and its alleviation in rice seedlings are rare.

The major objective of the present study was to investigate the Cr^{+6} induced changes in growth, pigment biosynthesis and proline content in developing rice seedlings grown under hydroponics. The study was assessed for different treatments of Cr^{+6} with and without application of chelating agents like EDTA, DTPA and EDDHA.

The study revealed the physiological and biochemical changes in the rice seedlings by supplementing chelating agents in the nutrient solution. The toxic behaviour of Cr^{+6} in presence of chelating agents during seedling growth of rice were also investigated through assessing chlorophyll and proline biosynthesis.

Chromium bioavailability in root and shoot system of growing rice seedlings have been undertaken in presence of chelating agents. In view of the toxic effects of Cr^{+6}, the present investigation is an attempt to study the role of different chelating agents with an aim for attenuation of Cr^{+6} in an *in situ* phytoremediation programme.

Materials and Methods

Plant Material

Dry graded uniform rice seeds (*Oryza sativa* L. var. Khandagiri) were procured from Central Rice Research Institute, Cuttack and were surface sterilized with 0.1% mercuric chloride (w/v) for 5 minutes. The seeds were germinated in petriplates containing saturated cotton pads with distilled water and kept in BOD incubator at 25 ±2 ^{0}C.

Growth of Rice Seedlings

On third day, the germinated seeds were transferred to thermocoal glasses containing Hoagland nutrient solution (half strength) and kept in growth chamber under continuous white light. White light was provided by cool fluorescent white tubes (36 W Philips TLD) with a photon flux density of 52 $\mu Em^{-2}s^{-1}$ (PAR) under 16h photoperiod. The seedlings were grown under different treatments viz. Cr^{+6}-EDTA (10µM), Cr^{+6}- DTPA (10µM), and Cr^{+6} -EDDHA (10µM).

Biochemical Analysis

Analysis of seedling growth, pigment content and proline accumulation was conducted using 9 days old rice seedlings. For analysis of chlorophyll, carotenoids and proline, the primary leaves of rice seedlings were taken. The extraction of chlorophyll and carotenoid was made using 80% cold alkaline acetone and calculated [1, 19]. Proline was estimated as per method of Bates *et al.,* [3].

Nine days old rice seedlings grown in different treatment of Cr^{+6} were analyzed for total Cr content in root, and shoots [4]. Before analysis of total Cr and total Fe content, the roots were rinsed with 0.01N HCl followed by washing with distilled water for removing mixed Fe and Cr hydroxides, which may have precipitated on the root surfaces.

Plant mass were analyzed for Total Accumulation Rate (TAR) and Transportation index (Ti) as per following method [27, 9].

$$TAR = \frac{(\text{Shoot concentration x Shoot biomass + Root concentration x Root biomass})}{[(\text{Shoot biomass+ Root biomass}) \text{ x days of growth}]}$$

N.B.: TAR (mg/kg/day), Biomass (gm dry wt.) and Concentration (mg/kg dry matter)

$$Ti = \frac{\text{Cr concentration of leaves (mg/kg)}}{\text{Cr content of root (mg/kg)}} \times 100$$

Statistical Analysis

All the treatments were conducted in triplicates each and the data presented in the figures and tables are mean ± SEM (Standard Error of Mean) of three replicates. One-way ANOVA was carried out to compare the means of the different treatments where F values were detected.

Results and Discussion

Growth Parameter Study

The growth parameter studies of rice seedlings were conducted for 5, 7, 9 and 11 days-old plants. A significant deterioration of seedling growth was found with increasing concentration of Cr^{+6} in nutrient solutions. But application of chelating agents (EDTA, DTPA and EDDHA) was advantageous for combating toxicological effects and stimulating growth in rice seedlings. The use of chelating agents induced better growth as compared to control and other kind of treatments (Cr-10µM and Cr-100µM) as shown in Fig. (**1**). Particularly, Cr^{+6}-EDDHA (10µM) induced better root length whereas Cr^{+6}-EDTA (10µM) showed maximum shoot length Fig. (**2 a & b**). The stimulation of growth was also accompanied by increased biomass content for above mentioned treatments Fig. (**3 a & b**). It has been inferred that root and shoot length of rice seedlings varies significantly (at $p \leq 0.05$ and $p \leq 0.01$) among the different treatments.

The effect of Cr^{+6} (100µM) on root was found to be highly toxic as the length of the roots have been significantly reduced as compared to control. Similar results have been reported by several other workers in other plants [25, 26, 17]. Hexavalent chromium affects plant growth and metabolism by decreasing nutrient uptake and photosynthetic abilities [4, 22,2]. Biomass content, root and shoot length of rice seedlings were significantly increased (at $p \leq 0.05$ and $p \leq 0.01$) when grown in nutrient solution supplemented with chelating agents. Rice seedlings grown under treatment of Cr^{+6}(100µM) showed very reduced growth indicating stunted plant height, decreased root and shoot length and much reduced biomass content in comparison to control and others.

The fresh and dry matter of seedlings during growth was illustrated in Fig. (**3**) and Fig. (**4**). A uniform trend was observed for length and biomass content of root and shoots of developing rice seedlings for 5, 7, 9 and 11 days growth period. Highest root/ shoot ratio was observed in the seedlings when Cr^{+6}-EDDHA (10µM) was supplemented in the nutrient solutions. Fresh and dry mass though decreased with increase in chromium concentrations exhibited a modulatory role as reported elsewhere [20].

Total chlorophyll content in the seedlings treated with Cr^{+6}-EDTA (10µM) solution was more as compared to other treatments Fig. (**5a**). The total chlorophyll content decreased with increase in Cr^{+6} concentrations. Chlorophyll and carotenoid contents reduced after 9 days growth of rice seedlings. It was highest at 9th day of seedling growth. Cr^{+6} at 100µM concentrations induced toxicity symptoms showing reduced chlorophyll biosynthesis which was attributed to reduced photosynthesis whereas Cr^{+6} at 10µM concentration stimulated chlorophyll biosynthesis. Carotenoid status showed similar trend of increased biosynthesis like chlorophyll Fig. (**5b**). The seedlings treated with chelating agents showed high pigment biosynthesis. Similar reports on the impacts of metal toxicity are available in different plants [23, 25]. The seedling carotenoids, on the other hand, decreased at 100µM concentration of Cr^{+6}. Similar events of reduced pigment biosynthesis have been reported under salt stress [10].

A marked increase in proline content was found in the seedlings grown under Cr^{+6} (100µM) treatments Fig. (**6**).

Proline accumulation is an important parameter to recognize the stress impact on plants [14, 24]. For example, stress induced proline biosynthesis was observed in many plants in response to salt stress [13, 16]. Proline accumulation may also help in nonenzymic free radical detoxifications [18; 7, 13].

Increasing proline level is considered to help the cells in osmoprotection as well as in regulating the redox potential, scavenging hydroxyl radicals and gives the protection against denaturation of various macromolecules [13]. In the present context, a uniform increase in the proline level was noticed in 9 and 11 days old rice seedlings subjected to Cr^{+6} (100 μM) treatment. An increase in proline content in rice seedlings as suggested above may be an osmoprotection to rice seedlings providing nontoxic sinks for carbon and nitrogen preservation as reported for other plants under salt stress [21, 11, 6].

Above physiological studies revealed that 9 days old seedlings showed better growth response and optimum biosynthesis as compared to 11 day old seedlings. Therefore, Chromium content was analyzed for 9 day old rice seedling. Table 1 illustrates the changes in the total Cr content in roots and shoots of 9 days old rice seedlings. There was an increase in the Cr content with increase in concentration in the nutrient solutions. Root chromium bioaccumulation was highest in the seedlings under the treatments of Cr^{+6} (100 μM). Application of EDTA in combination with Cr^{+6} (10μM) showed better root Cr bioaccumulation in rice seedlings. Shoot uptake of Cr was high in rice seedlings when treated with Cr^{+6}-DTPA (10μM).

Transportation index (Ti) gives the leaf/root chromium concentration and depicts the ability of the plant to translocate the metal species from roots to leaves and compartmentalize it at different concentrations [9]. Supplementation of Cr^{+6}-DTPA (10μM) in nutrient solution increases the translocation of Cr from root to shoot which eventually helps in continuous phytoextraction process. High values of Transportation index (Ti) reduce the Cr concentration in the nutrient solution and thus lessen the toxicity potential to roots. It also increases the resistance mechanism of plants to high Cr concentration. So addition of chelating agents enhances the *in situ* phytoremediation ability of the plant. Total Accumulation Rate (TAR) was high in rice seedlings growing in nutrient solutions supplied with Cr^{+6}: EDTA (10μM).

The high degree of Cr bioaccumulation, increased proline biosynthesis and stunted seedling growth with low pigment status indicated a positive Cr^{+6} induced toxicological effects in developing rice seedlings. To combat against such toxicity effects application of chelating agents could be an effective measure. The study reveals the beneficial and effective role of chelating agents in stimulating plant growth and metabolism. These chelated chromium compounds can overcome the toxicity problems of chromium to a greater extent for which further elaborate study is warranted.

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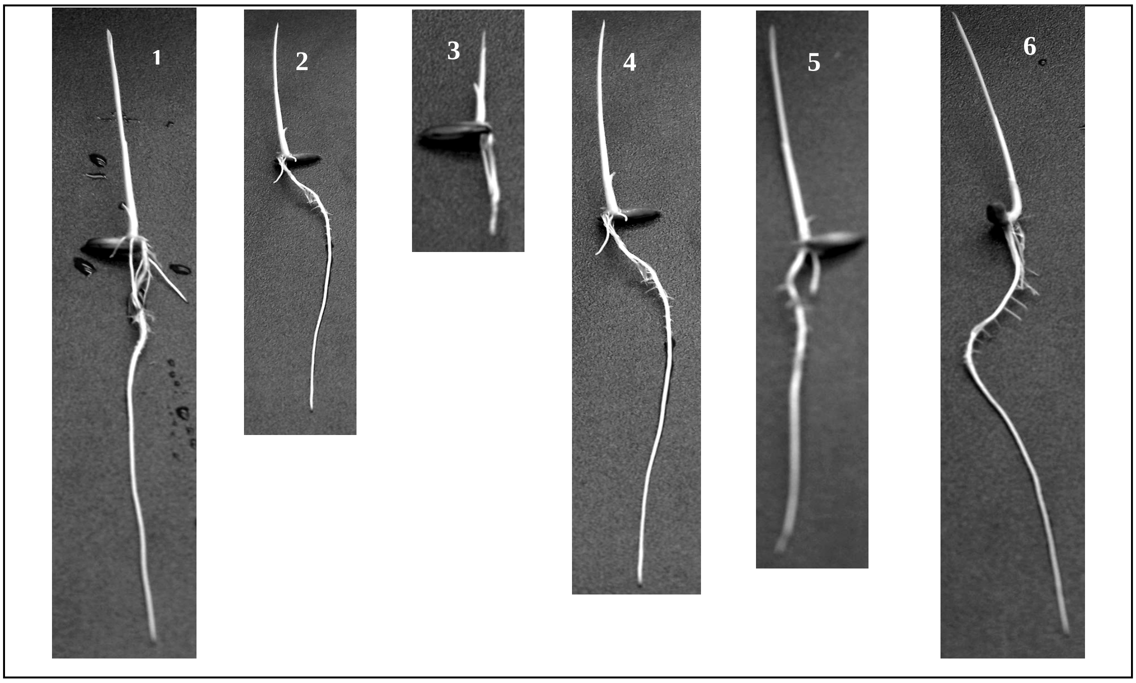
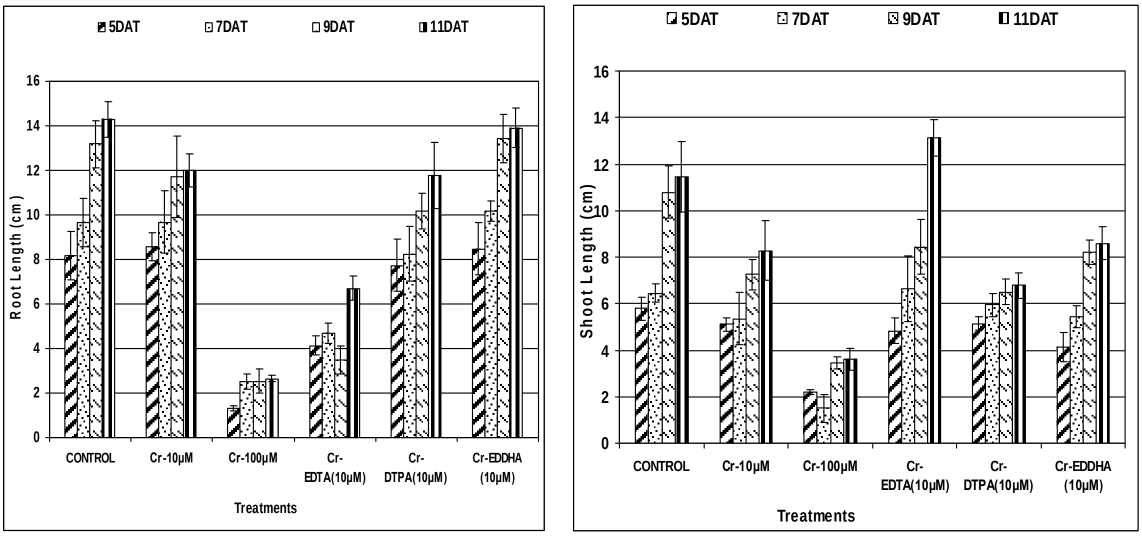
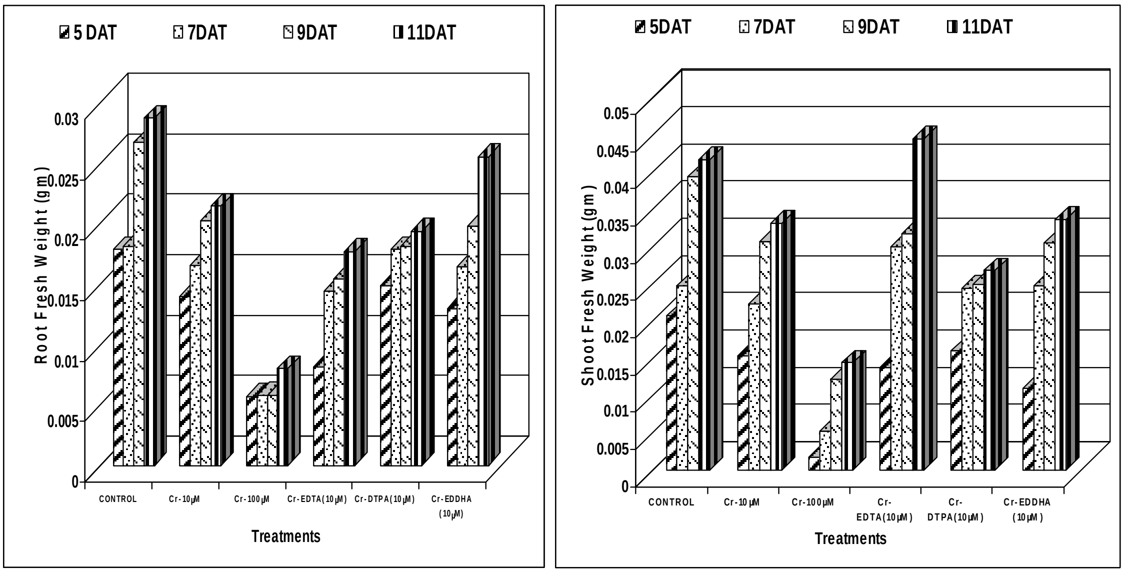


Fig. 1- Five days old rice seedlings showing root and shoot growth in different treatments of Cr^{+6} and chelating agents. [No.1: Control; No.2: Cr^{+6} (10µM); No.3: Cr^{+6} (100µM); No.4: Cr^{+6}–EDTA(10µM); No.5: Cr^{+6}–DTPA (10µM);No.6: Cr^{+6}-EDDHA(10µM)].



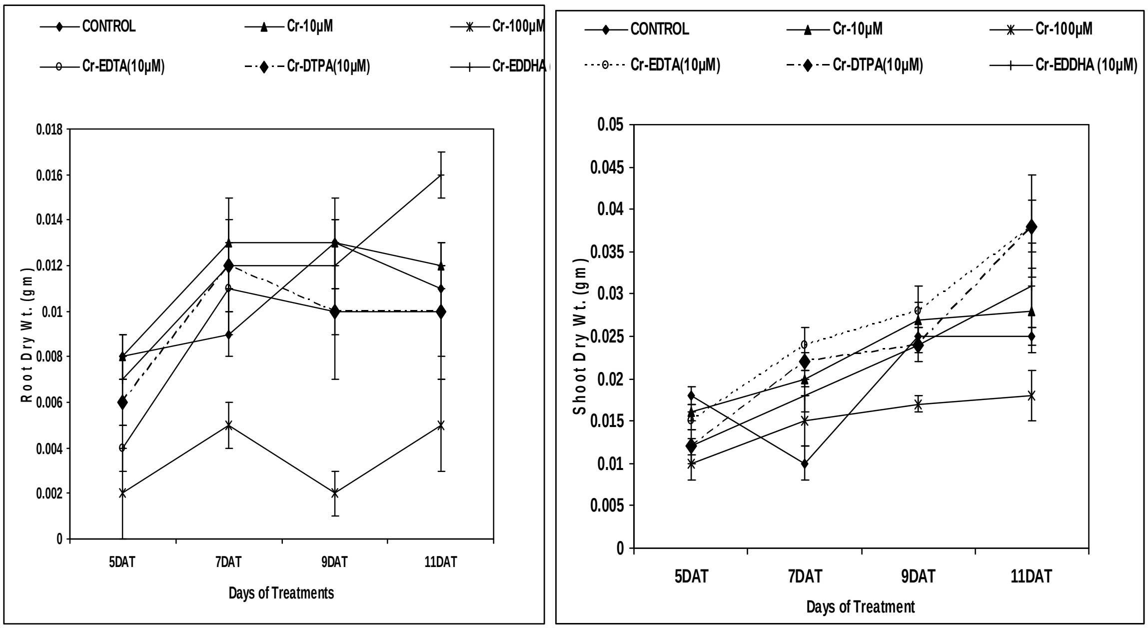
a b

Fig. 2- (a&b). Effect of Cr^{+6} and chelating agents on root (a) and shoot length (b) of 5, 7, 9 and 11 days old rice Seedlings. (DAT: Days After Treatment) Data represents Mean±SEM.



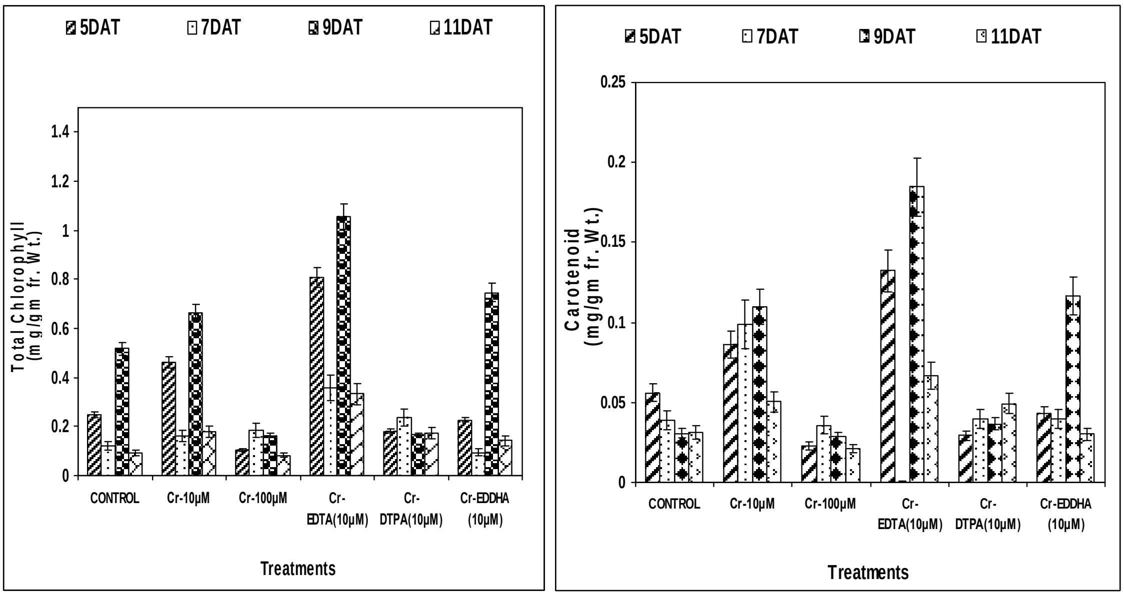
a b

Fig. 3- (a&b). Effect of Cr^{+6} and chelating agents on root (a) and shoot fresh matter (b) of 5, 7, 9 and 11days old rice seedlings. (DAT: Days After Treatment) Data represents mean±SEM.



a b

Fig. 4-(a&b). Effect of Cr^{+6} and chelating agents on root (a) and shoot dry matter (b) of 5, 7, 9 and 11 days old rice seedlings. (DAT: Days After Treatment) Data represents mean±SEM.



(a) **(b)**

Fig. 5- (a&b). Effect of Cr^{+6} and chelating agents on total chlorophyll (a) and carotenoid (b) content of 5, 7, 9 and 11 days old rice seedlings. (DAT: Days After Treatment) Data represents mean±SEM

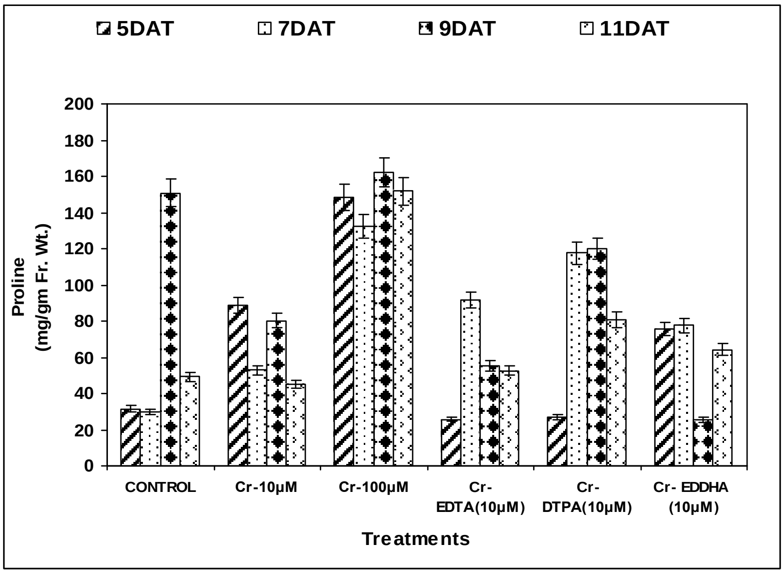


Fig. 6-Effect of Cr^{+6} and chelating agents on proline content of 5, 7, 9 and 11 days old rice seedlings. (DAT: Days After Treatment). Data represents mean±SEM.

Table 1- Effect of Cr^{+6} and chelating agents on chromium bio-availability (mg / Kg dry matter) in roots and shoots, Ti and TAR of 9 days old rice seedling grown in hydroponics (Values are mean ± SEM of three replicates).

Treatments	Chromium Content (mg / Kg dry matter)		Transportation Index (Ti)	Total Accumulation Rate (TAR) (mg/kg/day)
	Root	Shoot		
Control	0.0001 ± 0.000	0.000 ± 0.000	0.000	0.000
Cr^{+6} (10µM)	58.052 ± 0.035	27.356 ± 0.289	47.123	0.006
Cr^{+6} (100µM)	98.95 ± 0.189	22.255 ± 0.108	22.491	0.003
Cr^{+6} : EDTA (10µM)	93.648 ± 0.302	12.712 ± 0.327	13.574	0.011
Cr^{+6} : DTPA (10µM)	76.230 ± 0.381	32.236 ±0.231	42.288	0.005
Cr^{+6} : EDDHA (10µM)	59.55 ± 0.174	15.469 ± 0.267	25.976	0.008