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ORIGINAL RESEARCH PAPER

Responses of endogenous proline in rice seedlings under chromium exposure

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ABSTRACT: Hydroponic experiments were performed to exam the dynamic change of endogenous proline in rice seedlings exposed to potassium chromate chromium (VI) or chromium nitrate chromium (III). Although accumulation of both chromium species in rice seedlings was obvious, more chromium was detected in plant tissues of rice seedlings exposed to chromium (III) than those in chromium (VI), majority being in roots rather than shoots. Results also showed that the accumulation capacity of chromium by rice seedlings was positively correlated to chromium concentrations supplied in both chromium variants and the accumulation curve depicted an exponential trend in both chromium treatments over the entire period of exposure. Proline assays showed that both chromium variants induced the change of endogenous proline in shoots and roots of rice seedlings. Chromium (VI) of 12.8 mg/L increased proline content significantly (p<0.05) compared to control, while the effect of chromium (III) on proline content was more evident at 30.0 mg/L (p<0.05). The results collected here suggest that both chromium variants are able to cause the change of endogenous proline in rice seedlings, but the response was found to be different between the two chromium treatments.

KEYWORDS: *Accumulation; Chromium; Proline; Rice; Translocation*

INTRODUCTION

A variety of abiotic and biotic factors adversely affect plant growth and development through multiple biochemical and physiological reactions in plant internal biosystems (Misra and Gupta, 2005; Khan *et al*., 2012). Therefore, plants have developed different adaptive strategies to adapt, tolerate and avoid these stressful conditions. Among these different survival strategies, accumulation of compatible solutes has been considered as one of the main adaptive mechanisms for plants (Claussen, 2005; Islam *et al*., 2009; Yu *et al*., 2013) under various stress conditions such as water shortage, salinity, extreme temperatures, high light intensity, pathogen infection, nutrient deficiency and heavy metal stresses (Delauney and Verna, 1993; Hare *et al.,* 1999; Mansour, 2000; Islam *et al*., 2009). It is suggestive that proline is the most widely accumulated cellular osmolyte that permit osmotic adjustment in plants (Kohan *et al*., 2012; Wang *et al*., 2015). In spite of its established role as an osmoprotectant, it has been documented that increased content of proline accumulated in plant materials can contribute to scavenging hydroxyl radicals, stabilizing cell membranes by interacting with phospholipids, protecting folded protein structures against denaturation, modulating cell redox homeostasis, operating as a signal molecule to interact with other biochemical process, and serving as an energy and nitrogen source under different stresses (Hasegawa *et al*., 2000; Mansour, 2000; Claussen, 2005; Sharma and Dietz, 2006; Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008; Islam *et al*., 2009; Sharma *et al*., 2012; Yu *et al*., 2013; Wang *et al*., 2015). Ample evidence

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showed that plants are exposed to heavy metals mainly from exogenous sources. In plants, heavy metals disturb and/or damage a variety of biochemical and physiological processes, resulting in lessen of plant growth, restriction of transpiration, induction of chlorosis, disturbance of antioxidant enzymes, damage of nutrient balance, and cell death (Carrier *et al*., 2003; Scoccianti *et al*., 2006; Wang *et al*., 2009). Chromium (Cr) is a non-essential element that is toxic to plants, even at low concentrations. It is obvious that Cr(VI) and Cr(III) are the two most common and stable chemicals in the family of Cr. Phytotoxicity of both Cr variants has been extensively studied in many plants (Vajpayee *et al*., 2000; Mei *et al*., 2002; Yu *et al*., 2010). However, little information is available on the relationship between endogenous proline and Cr accumulation in rice seedlings. Additionally, the cation species of Cr(III) has a positive charge of +3, while the anion Cr(VI) has a negatively charged function group of CrO_4^{2-}. Such a difference might cause different uptake mechanisms by plants, consequently resulted in different metabolic responses between the two Cr variants. The objectives of this study were to investigate whether proline accumulated in rice seedlings exposed to Cr(VI) or Cr(III) and clarify whether any accumulation was concentration dependent. The dynamic changes of endogenous proline in rice seedlings exposed to both Cr variants and the responsive difference between the two Cr variants were determined.

This study was carried out at the College of Environmental Science and Engineering, Guilin University of Technology, P. R. China from October 2015 to April 2016.

MATERIALS AND METHODS

Preparation of rice seedlings

Preparation of rice seedlings was identical to our previous investigation (Yu *et al*., 2015). Briefly, seeds of rice (*Oryza sativa* L. cv. BX139) were planted in sandy soils in a climate control chamber at 25°C, and only added with nutrient solution (2823.9 mM KNO_3, 59.0 mM $MgCl_2.6H_2O$, 122.4 mM $CaCl_2.2H_2O$, 60.9 mM $MgSO_4.7H_2O$, 246.0 mM KH_2PO_4, 10.0 mM, Fe-EDTA, 2992.1 nM, H_3BO_3, 2097.0 nM, $MnCl_2.4H_2O$, 22.0 nM $ZnCl_2$, 6.3 nM $CoCl_2.6H_2O$, 0.1 nM $CuCl_2.2H_2O$ and 28.9 nM $NaMoO_4.2H_2O$) (Zhang *et al*., 2014). After 16 d of growth, young seedlings with similar height and weight were collected and rinsed with deionized water. In order to eliminate the ions from the cell wall space, pre-selected rice seedlings were grouped and kept in a *washing* solution containing 1 mM $CaCl_2$ + 2 mM MES-Tris (pH 6.0) for 4 h (Ebbs *et al*., 2008). After cleaning, all seedlings treated were used for the subsequent experiments.

Determination of Cr in rice seedlings

Approximately one gram of pre-treated young seedlings were exposed to 50 mL Cr-spiked solution containing 1.60, 6.40 and 12.8 mg Cr/L Cr(VI) or 3.0, 12.0 and 30.0 mg Cr/L Cr(III). Potassium chromate (K_2CrO_4) and chromium nitrate ($Cr(NO_3)_3.9H_2O$) of analytical grade with e” 95% purity was used. Exposure regime was similar to those described previously (Yu *et al*., 2014). Flasks with seedlings were placed in a climate control chamber under continuous artificial light (illumination intensity: 20,000 lux). Temperature was kept at 25 ± 0.5 °C. Relative humidity was set to 60 ± 2%. After exposure of each 24 h, seedlings collected were rinsed with deionized water and separated into roots and shoots. After drying at 90 °C for 2 d, plant samples were acid-mixed with 8 mL of 4:1 HNO_3-$HClO_4$ solution for 12 h (Zhang *et al*., 2014). Then, samples were acid-disguested at 200 °C for 2 h. The cooled residue was transferred into 50 mL glass flasks and added 0.2 mL 1% HNO_3. Finally, the solution was made up to 50 mL with deionized water. The total Cr concentrations in different parts of rice seedlings were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; PerkinElmer NexION 300X, USA) at intervals of 24 h over a period of 120 h. The detection limits, determined as mean blank plus three times the standard deviation of ten blanks, was 0.005 mg Cr/kg DW for plant materials. The sample preparation methods used were also checked against samples spiked with certified solution standards; mean recovery was 96.49%. The precision of Cr determination, based on variations of replicate analyses (n = 2) for the same sample, was <15%.

Determination of proline in rice seedlings

A slight modified procedure (Li, 2006; Kumar *et al*., 2013) was used to determine the proline content in plant tissues. After exposing to Cr(VI) or Cr(III), roots and shoots separated from rice seedlings were washed with deionized water. 0.5 g of plant tissues, after homogenizing with 5 mL of 3% aqueous sulfosalicylic acid on ice bath, was used for proline determination.

The extracts were kept at 100 °C for 10 min. After cooling, centrifugation was performed at 3000 ´ *g* for 10 min. The reaction mixture contained 2 mL glacial acetic acid, 2 mL of freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid) and 2.0 mL of supernatant. Again, the mixture was heated at 100 °C for 30 min for color development, and then cooled to room temperature before being extracted with 4 mL of toluene. The absorbance was monitored at 520 nm against toluene as a blank using a SHIMADZU UV-2410PC spectrophotometer. The proline content in different tissues of rice seedlings were measured at intervals of 24 h over a period of 120 h.

Statistical analysis

Significance analysis at 0.05 between the variables was conducted using Tukey's multiple range test (Zar, 1999). The SPSS (version 20) was used for the statistical analysis of data.

RESULTS AND DISCUSSION

Exposure of rice seedlings to Cr(VI)

Rice seedlings were grown in the Cr(VI) spiked hydroponics for 120 h. Changes of total Cr concentration (mg/g DW) in plant tissues of rice seedlings exposed to Cr(VI) with time (h) are given in Fig.1. The Cr concentrations in roots and shoots from un-treated seedlings were below the detection limit, while significant amounts of Cr was detected in both parts of rice seedlings from all treatment groups with Cr(VI), indicating uptake and translocation of Cr(VI). At the treatment of 1.6 mg Cr/L, the total Cr concentration in roots (Fig. 1a$_1$) increased from 372.74 to 615.07 mg/g during the 120-h period, and more for higher Cr(VI) treatments (Fig. 1a and b). A similar accumulation trend was also observed in shoots, but amounts of total Cr accumulated in shoots were significantly lower than those in roots (p<0.05). Indeed, Cr concentration in shoots (Fig. 1a) increased from 47.22 to 55.51 mg/g at 1.6 mg Cr/L during the 120-h

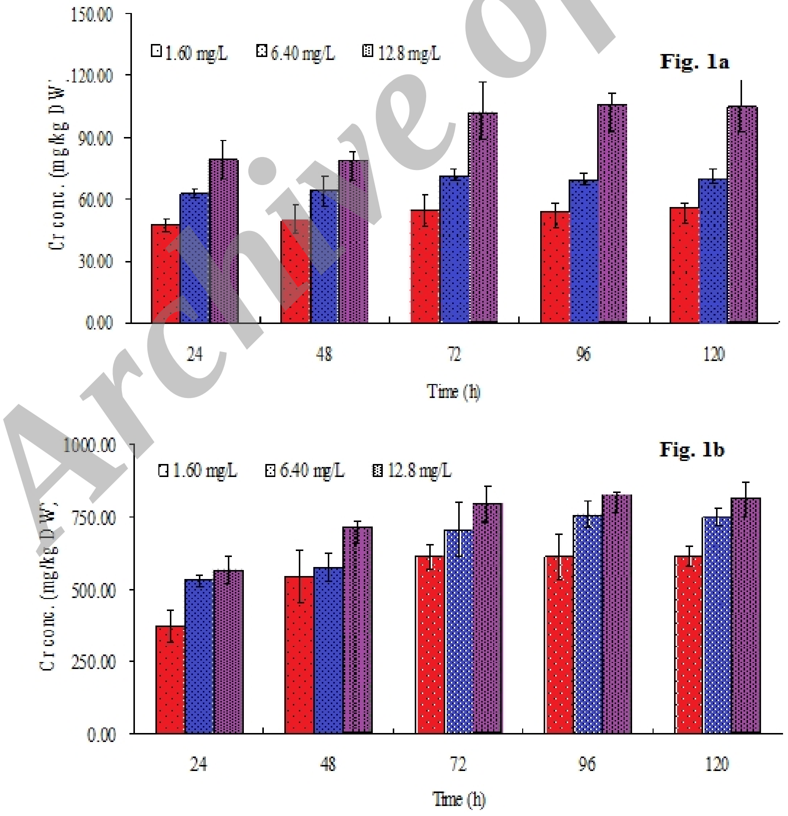

Fig. 1: Measured total Cr concentrations in shoots (**a**) and roots (**b**) of rice seedlings exposed to Cr(VI) over time
The values are the mean±SD (standard deviation) (n=4)

period, while more Cr was detected in higher Cr(VI) treatments ($p<0.05$). It can be observed in Fig. 2a (shoot) and b (root) that the Cr accumulation curve had an exponential trend at the Cr(VI) treatment of 1.60 mg/L. Obviously, rice seedlings showed a great capacity for accumulating Cr(VI) in biomass in the first 72 h afterward more gradual, while it reached almost unchanged at 96 h.

Response of endogenous proline in rice seedlings to Cr(VI) exposure

In control (without application Cr), negligible change of proline concentration was detected in both root and shoots of rice seedlings exposed to Cr(VI) over a 120-h period of exposure (Fig.3a and b), however significant higher proline concentration was found in shoots (Fig. 3a) (mean 9.65, SD 0.31, no=5) than roots (Fig.3b) (mean 7.52, SD 0.48, no=5). Proline analysis also showed that all Cr(VI) treatments induced the proline changes in both materials. At the treatment of 1.6 mg Cr/L, there was a marginal increase in the accumulation of proline in both plant materials (shoot: mean10.65, SD 0.45, no=5; root: mean 7.82, SD 0.54, no=5) over the 120-h period in comparison to control ($p>0.05$). A moderate increase in proline level was observed in both roots and shoots of rice seedlings exposed to 6.4 mg/L ($p>0.05$). However, significant increase (shoot: mean15.68, SD 1.86, no=5; root: mean 9.80, SD 1.20, no=5) was detected at the 12.8 mg/L treatment ($p<0.05$). It is also interesting to note, in both plant materials, that the content of proline enhanced quickly during the first 48 h exposure, and decreased gradually in all Cr(VI) treatments.

Exposure of rice seedlings to Cr(III)

An increase trend in total Cr concentrations was observed in all Cr(III) treatments with increasing exposure time (Fig.4a and b). A rapid increase in Cr content was detected in both roots and shoots of rice seedlings exposed to Cr(III) at 24-76h. For example, Cr concentration in roots (shoots) increased from 1081.32 (61.81) to 1320.86 (73.91) mg/g at 3.0 mg Cr/L (Fig.4a and b) during the 72-h period, and almost constant afterwards (72-120 h). Similarly, an exponential trend in Cr accumulation was observed in all Cr(III) treatments (Figure not shown).

Response of endogenous proline in rice seedlings to Cr(III) exposure

Negligible change of the proline content was observed in both plant materials (shoot: mean10.28, SD 0.61, no=5; root: mean 7.79, SD 0.48, no=5) at 3.0 mg Cr/L treatment (Fig. 5a and 5b) over the 120-h period in comparison to control ($p>0.05$). At the treatment of 30.0 mg/L, significant increase (shoot: mean12.99, SD 1.67, no=5; root: mean 10.73, SD 1.49, no=5) was detected ($p<0.05$). In both plant materials of rice seedlings exposed to Cr(III), the accumulation of proline increased quickly during the first 72 h exposure, and decreased gradually.

It has been reported that both Cr(VI) and Cr(III) variants can be easily taken up by plants (Vajpayee *et al.*, 2000). Results of the present study strengthen the earlier view that rice seedlings were able to accumulate

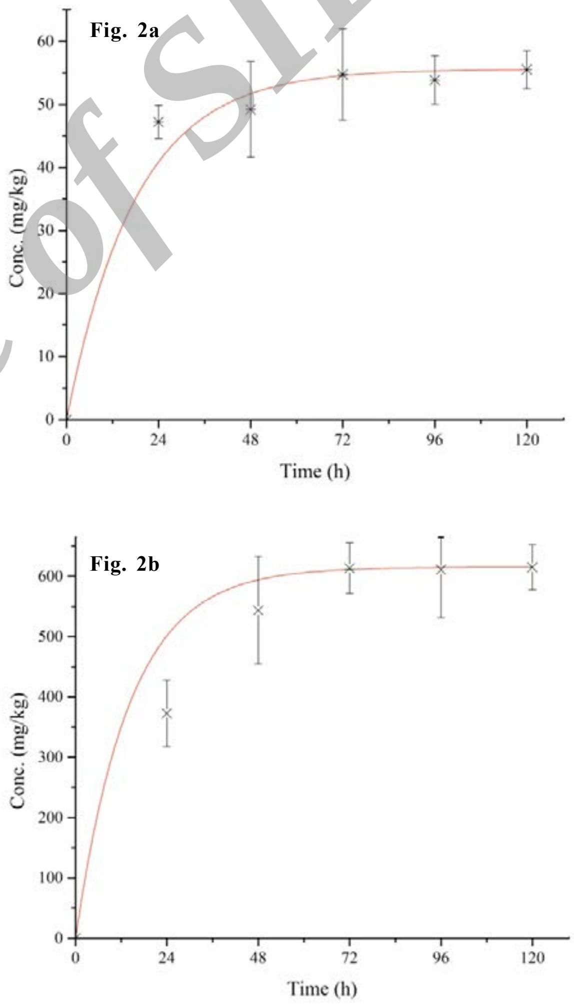

Fig. 2: Examples of exponential curves for the Cr accumulation in shoots (**a**) and roots (**b**) from the Cr(VI) treatment of 1.60 mg/L

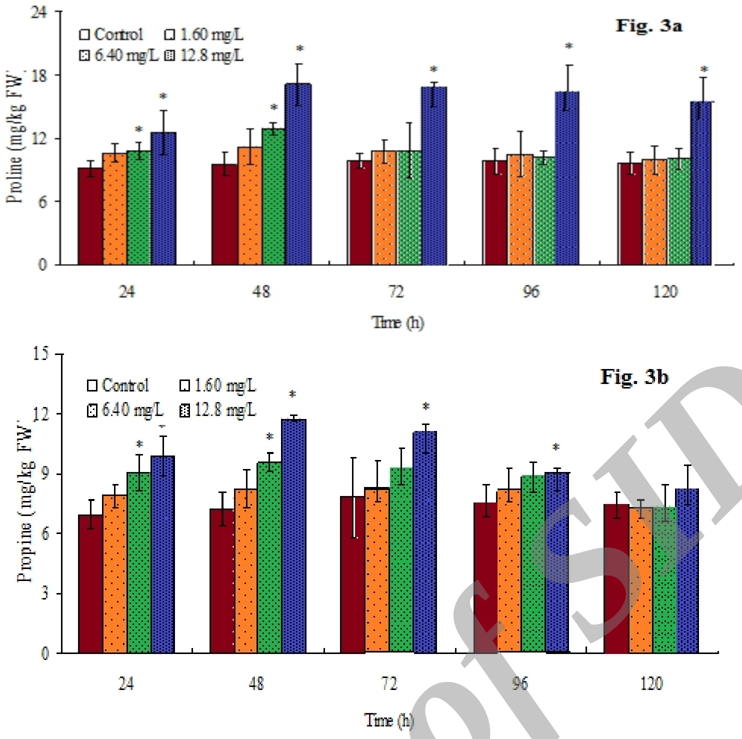

Fig. 3: Measured proline content in shoots (**a**) and roots (**b**) of rice seedlings exposed to Cr(VI) over time
The values are the mean±SD (standard deviation) (n=4). Bars with asterisk symbol refers to significant level in comparison to control (p<0.05)

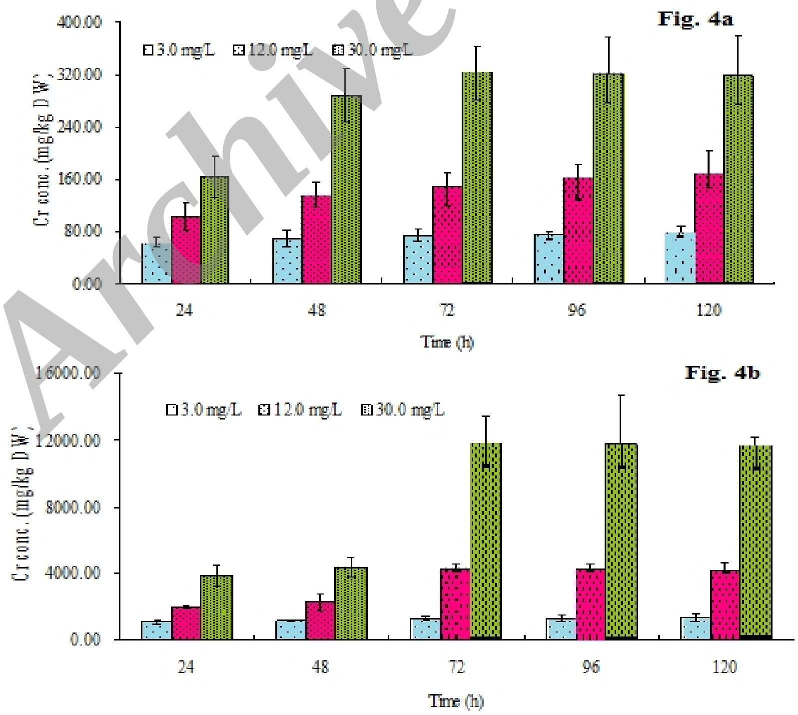

Fig. 4: Measured total Cr concentrations in shoots (**a**) and hoots (**b**) of rice seedlings exposed to Cr(III) over time
The values are the mean±SD (standard deviation) (n=4)

both Cr variants in various tissues of plants. However, different plant materials accumulated Cr in varying amounts (roots > shoots). The present study also found that more Cr accumulation was evident in plant materials of rice seedlings exposed to Cr(III) than those in Cr(VI) treatments ($p < 0.05$), suggesting that Cr(III) is more bioavailable for plants than Cr(VI). Similar conclusions were also reported by Mei *et al*., (2002) and Yu *et al*., (2010) previously.

However, the biological fate of both Cr variants in plant materials seems to be different, judged by the translocation factor (TF), which is the fraction of Cr in the shoots to roots (Ren *et al*., 2014). In the study the TF of both Cr variants were calculated. Rice seedlings showed ineffective capacity of translocating either Cr(VI) or Cr(III) as the highest TF was 0.127. However, TF values for Cr(VI) was 2.0 time higher (0.097-0.127) than Cr(III) (0.027-0.066), suggesting that Cr(VI) was more mobile than Cr(III). The authors also noted that TF values remained almost unchanged in Cr(VI) treatments during the entire period of exposure. The change of TF values between the three Cr(III) treatments were quite different. A constant TF value (mean 0.057, SD 0.001) was obtained in the Cr(III) treatments of 3.0 mg/L, where the TF values in other two Cr(III) responded biphasically to the duration of exposure by showing increases at 48 h and constants afterwards, suggesting ineffective translocation capacity of Cr(III) by rice seedlings.

Our results showed that the change of endogenous proline was responsive to both Cr concentrations. However, the response between the two Cr variants was quite different. Based on the current results, the linear plot between the proline content and Cr supplied was conducted (Figure not shown). For the Cr(III) treatment, both linear regressions for root and shoots were significant (roots: $y=0.1082x+7.5657$, $R^2=0.9998$; shoots: $y=0.1071x+9.8322$, $R^2=0.9895$), judged by the higher regression coefficient R^2. It is interesting to calculate the variation factor of Cr concentration supplied to the proline content accumulated between roots and shoots using the slope of the linear plot. The variation factor between shoots and roots of Cr(III)-treatment rice seedlings was almost equal to 1,

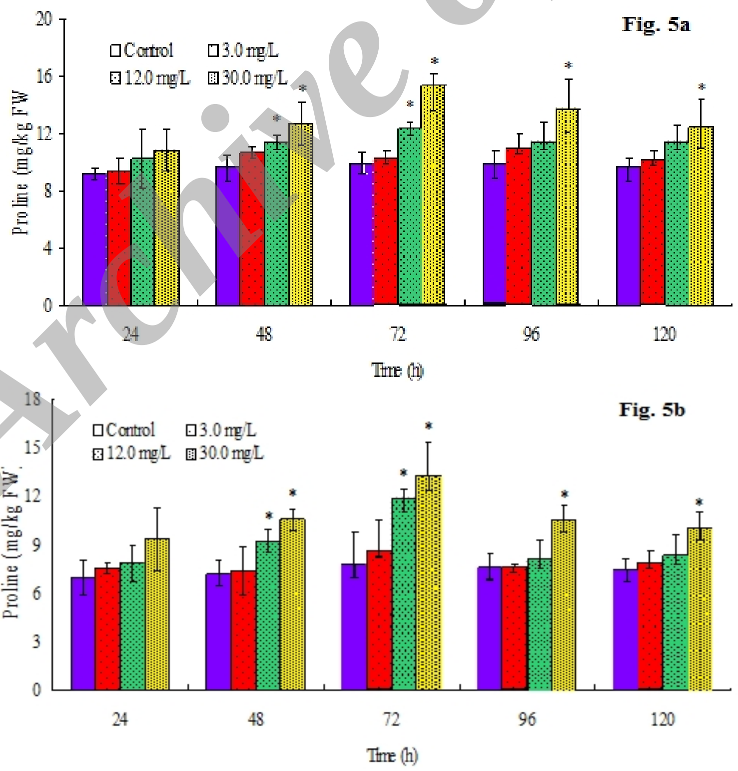

Fig. 5: Measured proline content in shoots (**a**) and roots (**b**) of rice seedlings exposed to Cr(III) over time
The values are the mean±SD (standard deviation) (n=4). Bars with asterisk symbol refers to significant level in comparison to control ($p<0.05$)

suggesting that the proline content from roots and shoots showed similar responses to Cr(III) exposure. Similar data analysis was used for Cr(VI) treatment. The outcome is quite surprising: both linear regressions were remarkable (the regression for roots y=0.1798x+7.5568, R^2=0.9946; the regression for shoot y=0.4415x+9.4425, R^2=0.8941). Thus, the difference between shoots and roots was 2.46, suggesting that the change of proline content in shoots to Cr(VI) exposure was more sensitive than to that in roots. Using the same statistical method, we also noted that the change of proline in rice seedlings was more responsive to Cr(VI) treatments than Cr(III) treatments.

CONCLUSION

Our results indicated that rice seedlings are able to remove both Cr(VI) and Cr(III) from the hydroponics. However, higher accumulation capacity for Cr(III) rather than Cr(VI) was observed. Distribution of Cr in plant tissue from both Cr treatments kept the same pattern, in which higher Cr accumulation in roots was detected than shoots. Both Cr(VI) and Cr(III) affected proline content in plant materials of rice seedlings, but the response between the two Cr treatments was different.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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