Journal of Experimental Biology and Agricultural Sciences, June - 2022; Volume – 10(3) page 511 – 523

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Phytoremediation of chromium, iron and nickel by Indian Rice Plant (*Oryza sativa* L.): An opportunity for management of multi-metal contaminated tannery wastewater

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Received – April 10, 2022; Revision – May 20, 2022; Accepted – June 17, 2022
Available Online – June 26, 2022

DOI: http://dx.doi.org/10.18006/2022.10(3).511.523

KEYWORDS

Phytoremediation

Oryza sativa

Heavy Metals

Translocation Factor

Phytoremediation

ABSTRACT

India is the largest producer of leather and leather products. Tannery industries use a large number of synthetic chemicals for the processing of leather and generate a huge amount of wastewater containing a large amount of potentially toxic heavy metals (PTHMs) making them problematic for next-door soil and water system. Currently, phytoremediation is an inexpensive green technology used to move, eradicate, and stabilized heavy metal contamination from contaminated sludge, soil, and wastewater. In this study, the accumulation and distribution of PTHMs found in tannery wastewater and their physio-biochemical effects on *Oryza sativa* L. have been studied by ICP-MS, GC-MS, and biochemical analysis. The plant was grown in the soil spiked with a mixture of metals (Cr, Fe and Ni) and their five-level of treatment T1 (25mg/kg); T2 (50mg/kg); T3 (100mg/kg); T4 (200mg/kg) and T5 (400mg/kg). During the experiments, various morphological attributes, oxidative stress, enzymatic activities, chlorophyll, and protein content at the different stage was measured. Further, metal accumulation pattern in different parts of plants was also measured. Results of the study revealed that plant root, shoot length, chlorophyll content, and enzymatic activities were significantly reduced after the treatment with 200 mg/kg PTHMs; whereas oxidative stress was increase compared to control levels. Further, treatment of PTHMs suggested that the rice plant (*Oryza sativa* L.) is well adapted to tolerate and accumulate a high level of heavy metals (up to 200mg/kg) in the root and shoot of the treated plants. If it is treated above this, then seeds were also affected and not safe for human consumption.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

In Asian countries, India is the foremost exporter of leather and its products. The tannery industries generate hazardous chemical waste due to the use of large amounts of synthetic chemicals to convert animal skins into the leather which are harmful to the environment. The released wastewater is characterized by high odors due to the presence of free ammonia, volatile organic compounds, and hydrogen sulphide (Senatore et al. 2021). Tannery wastewater (TWW) also has a high level of potentially toxic heavy metals (PTHMs) like Cr, Cd, etc., this mixture of heavy metals makes the tannery wastewater, problematic for the environment (Urbina-Suarez et al. 2021; Ahmed et al. 2022). Water/soil contaminated with these toxic heavy metals has become a worldwide concern due to their non-specific toxicity and long-lasting exposure to the environment. The accumulation of heavy metals in the environment is also contaminating the food chains. The toxicity of these PTHMs is non-specific and affects organisms via several competing pathways (Lakshmi et al. 2021).

A wide range of chemical, physical and biological treatment policies are being implemented for the reduction of PTHMs contamination from soil or water. Some of the conventional treatment methods are chemical fixation, soil replacement, and thermal desorption (Hansen et al. 2021). In addition, conventional treatment approaches are difficult to operate, as they are quite expensive, laborious, and produce a massive amount of secondary waste that requires further treatment. Therefore, there is need for a suitable treatment technique with minimal waste generation is required for the remediation of PTHMs present in TWW.

Recently, some plant species were used for phytoremediation techniques because they have the capabilities to accumulate PTHMs in their roots and shoot system (Diarra et al. 2021). This technique is an eco-friendly, emergent, and broadly accepted plant-based technology for the removal of PTHMs in polluted water or soil. A range of crops like *Brassica napus, Solanum nigrum,* and *Zea mays* have been discovered which accumulate and extract the residual amount of PTHMs from the soil or water. Gupta and Sinha (2007) evaluated the phytoextraction capability of different plants growing in tannery sludge dumping areas. According to Evangelou et al. (2015), few trees such as Willow (*Salix* spp), poplar (*Populus* spp), and few plants like maize (*Zea mays*), Indian mustard (*Brassica juncea*), have been known as a suitable candidate for the management of these heavy metals (Chowdhary et al. 2018). These plants have some advantages over other crops such as rapid growth, tolerance to harsh environmental conditions such as high salinity, aesthetically pleasant, control of soil erosion, high biomass production, and removal of heavy metal discharge (Lakshmi et al. 2021). The efficiency of rice plants in phytoremediation of PTHMs from water or soil has been reported by various researchers (Liu et al. 2007; Evangelou et al. 2015; Pajević et al. 2016; Yan et al. 2020). Further, Rice is being used in numerous research due to its vast range of adaptability to different environmental conditions and potential to tolerate abiotic stress (Liu et al. 2007; Bharagava et al. 2008; Satpathy et al. 2014; Olawale et al. 2021; Lakshmi et al. 2021). Earlier, the potential of the rice plant to remediate heavy metals like Cd, Pb, and Fe contaminated soil and water have been studied by various researchers (Liu et al. 2007; Satpathy et al. 2014; Olawale et al. 2021). India has high production of rice and many farmers irrigate their crop plants with industrial wastewater having a high load of toxic compounds including PTHMs due to the non-availability of alternative sources of irrigation. In the current study, the rice plant was selected for phytoremediation because the information related to the potential of rice plant in phytoremediation of two or three heavy metals is limited. The accumulation of heavy metals in crop plants can be health hazardous. Therefore, it is obvious that metal stability of experimental plant must be under continuous surveillance because the accumulation of heavy metals in crop plants may be hazardous for health and ecosystem. Thus, in this study, the effect of PTHMs contaminant water irrigation on rice plant root and shoot growth, biochemical changes, oxidative stress, assessment of accumulation of heavy metals, and heavy metal concentrations in seeds were evaluated to determine the safe limit of rice plant irrigation.

2 Materials and Methods

2.1 Description of sampling site and sampling

The TWW samples were collected from M/s Unnao tanneries pollution control company, A-7, Site-II, UPSIDC, Industrial Area. Unnao, 209101, U.P., India, in a pre-sterilized container of 10 litre capacity. Further, the estimation of heavy metal and physio-chemical analysis of the collected water samples has been carried out as per the standard method of APHA (2005; 2021). All the observations were recorded in triplicate and their mean values are reported with their variation. The pH, Electrical Conductivity (EC), and other ions of collected tannery effluent were measured by Orion 960 titrator (Thermofisher, USA) (Singh et al. 2015), while COD, BOD, and color were measured by open reflex method, 5-day method, and cobalt platinum method respectively (APHA 2005; 2021). Further, phosphate and sulphate concentrations were analyzed by the stannous chloride method and gravimetric method respectively (APHA 2005; 2021). The concentration of different toxic metals was measured by using Electron Model IRS intrepid II Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermofisher, USA)(Singh et al. 2015). The organic matters were detected by GC-MS (Agilent, USA).

2.2 Pot Experimental Design

The experiment was carried out during the rainy seasons (i.e. rice growing season from May to -October). Rice variety MTU1010 was selected for this experiment because of its largest production area in India. Only those heavy metals (Cr, Fe, and Ni) were selected for the experiment which has been detected in high load in tannery effluent samples; these were prepared at five different levels i.e. T1 (25mg/kg); T2(50 mg/kg); T3(100 mg/kg); T4(200 mg/kg) and T5(400 mg/kg). The stock solution was prepared with their respective salts such as potassium dichromate ($K_2Cr_2O_7$), ferric chloride ($FeCl_3$,) and nickel chloride ($NiCl_2$), and dissolved in 500 mL water. The unspiked/unexposed and uncontaminated air-dried agriculture field soil collected from the college campus was used for this experiment. In each pot, 10kg of collected soil was filled. The physicochemical analysis of this agricultural soil was carried out as per the standard protocol given in APHA (2021). The concentrations of heavy metals in soil were measured using an ICP-MS (Inductive Coupled Plasma Mass Spectrophotometer) Thermo, Electron Model IRS intrepid II, USA) and organic matters by GC-MS (Singh et al. 2015). Each earthen pot was treated with predefined concentrations of selected heavy metals in 5 replicates.

In this experiment, tap water was used as a control. The seeds of rice were dipped in a water bath for approximately 48 h at room temperature. For germination, seeds were kept under moist conditions (to maintain moisture these seeds are covered with moist two-layer cloth gauze) at room temperature for an additional 30 h and the sprouted seeds were grown in the paddy seedbed under field conditions and raised for seedlings. After 30 days, the seedlings were transplanted into the heavy metal contaminated soil containing pots @ 4 plants per pot. During the whole growth period, water in the pot was maintained 3-4 cm from the soil surface. The experiment was laid out as a Complete Randomized Block Design (CRBD) with five replicates. The harvesting data were recorded after the 4 months of transplantation.

2.3 Measurement of plant growth parameters

Plant samples were taken three times during the time course of this experiment, first: just before the plantation of rice seedlings to the pot (1^{st}month); second, after the 2.5^{th} month and third before harvesting the plant (4^{th} month). Collected plants were uprooted and washed off with running water followed by tap water to remove any contiguous particles. Further, these plants were rinsed with 20 mM calcium chloride solution along with distilled water for further analysis such as root, shoot growth, and lipid peroxidation (Singh et al. 2015).

Physio-biochemical analysis of root and shoot growth parameters were measured as per standard protocol (Singh et al. 2015). The fresh weights (FW) of root, shoot, and panicle were determined through an electronic weighing balance. All the plant samples were oven dried for 48 hrs at 70°C and cooled down to room temperature and dry weights (DW) were determined. Water content (WC %) was calculated as per the formula:

$$\mathbf{WC} = \frac{(\mathbf{FW} - \mathbf{DW})}{\mathbf{FW}} \times \mathbf{100}$$

Here DW = Dry Weight, FW = Fresh Weight; WC = Water Content

The pigments of selected plants i.e. Chlorophyll a, chlorophyll b, and carotenoids were determined by the spectrophotometer method as per the standard protocol given by Lichtenthaler and Wellburn (1983). The protein concentration was estimated by the Lowry method (Lowry et al. 1951).

2.4 Lipid peroxidation (LiP)

Lipid peroxidation (LiP) in plant samples was estimated indirectly in terms of Malondialdehyde (MDA). The total MDA content was estimated by the method of Heath and Packer (1968). In brief, 0.5 gm leaf and root tissues (air dried) crushed and homogenized with 2 mL of 0.25% (w/v) TBA (2- Thiobarbituric acid) in 10% (w/v) TCA (Trichloroacetic acid). Grinding of plant sample was done in a circular motion until small bubbles appeared. Finally, all homogenate was made up to the volume 4 mL with 0.25% (w/v) TBA in 10% (w/v) TCA. Samples were heated at 95°C for 30 minutes in the water bath. After cooling with ice, samples were transferred into fresh centrifuge tubes with proper shaking and centrifuged at 12000 rpm for 5 minutes. The final concentration of MDA was estimated by deducting the absorbance of supernatant (600nm) from an absorbance coefficient (532 nm) of 155 nmol cm^{-1}.

2.5 Preparation of plant enzyme extract for antioxidant activity

For the enzyme extract, 0.5gm of fresh leaf samples were selected and crushed in 4 mL of phosphate buffer (100 µM; pH 7.5) containing 1 mM EDTA and a tiny pinch (~50mg) of polyvinylpyrrolidone (PVP). This homogenate was subjected to centrifugation at 12000 rpm for 10 min at 4 °C and the crude supernatant was used for the quantification of antioxidant enzymes (Sarker and Oba 2018).

2.6 Antioxidant activity

Superoxide dismutase (SOD) activity was estimated as per the earlier published method of Nishikimi et al. (1972) with some modifications. In brief, the test was carried out in two steps. In step one, 1.1 mL pyrophosphate buffer (PPB), 0.2 mL Nitro Blue Tetrazolium (NBT), 0.2 ml PMS, and 20 µL enzyme extract were taken. While in the second step all the above reagents except the enzyme source have been used. The reaction was started simultaneously in both steps by the addition of 0.2 mL NADH.

After 90 sec, for checking the reaction,0.5 mL glacial acetic acid was added to each tube. The absorbance (560nm) of both set of tubes was recorded against a reagent blank. The difference between reference and experimental absorbance gives the inhibition of NBT reduction by enzyme source. The unit of SOD enzyme activity was defined as "the amount of enzyme required inhibiting the optical density (560 nm) of NBT reduction by 50% in one minute under the assay conditions". The results were expressed as a 1U (1 Unit) enzyme.

Catalase (CAT) activity was calculated by the method of Maehly and Chance (1954). Phosphate buffer (2mL) and 1 mL of diluted H_2O_2(0.2 M) were taken in a cuvette, and add 0.02 mL enzyme source in it and mixed carefully. The reduction in absorbance (at 240 nm) was recorded after every 30 seconds for up to 3 minutes against the negative control.

2.7 Analysis of heavy metals in different parts of plant

The plant samples were harvested at the end of the experiment. Plants were chopped into roots, shoots, and seeds and left to oven dry at 50 °C for one week. A crushed form of dry plant matter was ashed in a muffle furnace at 500 °C for 6 hours. Now, 1 gm of ash from all the samples were digested separately in a 100 mL beaker with a digestion mixture (nitric acid and perchloric acid 5:1 ratio) until white fume comes (Singh et al. 2015; Ranieri et al. 2021). Washed down the wall of a beaker with a minimal amount of distilled water and then filtered. Transferred filtrate made up to 20 mL in the volumetric flask as per standard protocol (APHA 2021). The concentration of various heavy metals was measured using an ICP-MS (Singh et al. 2015).

2.8 Metal bioaccumulation studies

Bioaccumulation factor (BAF) is defined as "the level of metal in an organism's tissue divided by its concentration in soil /water which is expressed in the equivalent unit". BAF was calculated by using the formula given by Yoon et al. (2006). Moreover, translocation factor (TF) was calculated for different metals because they played a significant role in different metabolic activities of plants. The TF was calculated as per the standard method given by Gupta and Sinha (2007).

2.9 Statistical Analysis

Kolmogorov Smirov test was used to test the normality of data. Numerical data were presented in the form of mean ±SD. A post hoc Tukey and least significance difference test (LSD) test followed by one-way ANOVA was applied to analyze the significant difference among sampling sites for different heavy metal and physico-chemical parameters; this post hock test was also applied in biochemical parameters during phytoextraction of heavy metals. Statistical analysis was carried out using the SPSS, version 22 (Chicago, USA). The level of significance means, the *p*-value was set as <0.05. Graphics were prepared with the help of Microsoft Excel and Statistical Software.

3 Results and Discussion

3.1 Chemical texture of TWW

The distributions of heavy metals in collected TWW samples were analyzed, and the results are summarized in Table 1. A varied range of metal concentration (mean ±SD) were found. A post hoc analysis showed that chromium (Cr) is a predominant metal in TWW (58±1.66 mg/L). This was followed by iron and its concentration in the untreated tannery effluent was 7.90±1.6 mg/L. The order of dominancy of heavy metals in tannery effluent is Cr>Fe>Ni>Zn>Mn>Cu>Pb. Further, tests on seasonal variation were found homogenous (p>0.05). The tannery industry is one of the pollutions generating industries that mainly causes metal pollution especially chromium in the environment (Appiah-Brempong et al. 2022). In India, there are more than 2000 tanneries, and over 80% of tanneries are engaged in the chrome tanning process (Saxena and Bharagava 2016). In the tanning process, the salt of chromium is used to convert hide into leather and eventually release a large amount of organic matter and heavy metals. Further, it was reported that chromium-containing wastewater causes a serious threat to soil and water pollution and poses a serious hazard to human health (Hansen et al. 2021; Ahmed et al. 2022). These industries discharge chromium-containing wastewater into the canal and river. Moreover, chromium toxicity depends on chemical specificity, thus the health related effect of chromium is concerned by the chemical form of exposure. It is well known that chromium (VI) is a potentially toxic substance to humans, animals, and microbes because it enters the cell via the surface transport system and is reduced to the level of chromium (III) which is associated with the breakdown of DNA (Shi et al. 2021; VonHandorf et al. 2021).

The mean pH value of untreated TWW was 8.0±0.6. The seasonal variation in pH and temperature was found to be homogenous (Table 1). The EC (μS/cm) of untreated TWW was 1323.29±25.7 in summer and 1343.42±30.54 in monsoon season (p>0.05) which exceeded than the Bureau of Indian Standard (BIS) permissible limit (>1000 μS/cm). Polyvalent ions such as calcium and magnesium are associated with the total hardness of the water. In this study, total hardness ranges between 1205±30.18 to 1312±29.41. Moreover, the presence of fluoride ions was not detected throughout the study. A significant level of nitrate, nitrite, and other pollution parameters like sulphate, phosphate, etc. was found. This may be due to nitrogen coming from animal skins that are processed in the tannery industry (Saxena and Bharagava 2016).

Table 1 Heavy metal and physio-chemical analysis of tannery wastewater

Heavy metals	Summer (in mg/L)	Monsoon (in mg/L)
Cr	59.04±2.04	57.31±1.31
Cu	0.202±0.01	0.26±0.02
Fe	7.904±1.6	7.84±1.84
Mn	0.28±0.02	0.23±0.01
Ni	2.16±0.31	2.31±0.24
Pb	0.04±0.04	0.06±0.02
Zn	1.04±0.12	1.15±0.21
Physic-chemical parameters	Summer	Monsoon
pH	8.0[a]±0.6	7.82[a] ±0.5
Temperature (°C)	27.3[a] ±1.02	26.8[a] ±1.1
Electrical Conductivity (µS/cm)	1323.29[a]±25.71	1343.42[a]±30.54
Alkalinity	2.20[a] ±0.35	2.87[a] ±0.81
Color (CU)	1124[a] ±11.2	1215[a] ±14.02
TS (mg/L)	9001[a] ±55.1	1942[a] ±38.64
TDS (mg/L)	727.8[a] ±33.3	6916[a] ±41.05
TSS (mg/L)	196.0[a] ±22.1	119.9[b] ±20.49
Total hardness	1205[a] ±30.18	1312[a] ±29.41
Fluoride (mg/L)	ND	ND
Nitrate (mg/L)	28.37[a] ±6.25	24[a] ±5.55
Phosphate (mg/L)	129[a] ±13.49	126.2[a] ±14.05
Sulphate (mg/L)	881[a] ±44.04	893.4[a] ±50.15
Chloride (mg/L)	123[a] ±11.9	134.0[a] ±10.19
BOD (mg/L)	4680[a] ±69.25	4123[a] ±61.25
COD (mg/L)	12510[a] ±200	12348[a] ±205
Nitrite (mg/L)	31.02[a] ±1.51	32.12[a] ±1.39

All values are in ppm except pH, Color and EC; ND- Not detectable; Different alphabets in row show significant variation (Student T test at $p<0.05$).

The chemical characteristics of TWW after conventional treatment has revealed that it has a high load of pollution parameters in terms of heavy metals as well as COD, BOD, phosphate, sulphate, and organic pollutant including heavy metals.

3.2 General characteristic of agricultural soil

The pH and organic matter are important controlling factors, which control the availability of metals to plant species. As soil pH and organic matter increase, heavy metal mobility gets decreases due to the precipitation of hydroxides, carbonates, and the formation of insoluble organic complexes (Javed et al. 2019). On the other hand, metals are more mobile at pH below 7 and moderate organic matter. In this study, the pH and organic matter of experimental soil was 6.7±0.15 and 1.52±0.48 %, respectively **(**Table 2**)**. These results are consistent with Kicińska et al. (2022) who suggested that heavy metal mobilization in the soil is a function of pH and organic matter. On other hand, the amount of PTHMs such as Cr, Cd, and Pb in agricultural soil was found to be almost negligible or below the detection limit. The general characteristics of experimental agriculture soil used in this study were presented in Table 2.

3.3 Effect of PTHMs levels on morphological parameters

Only three PTHMs Cr, Fe, and Ni were found in significant quantities in TWW. Therefore, further phytoremediation studies were carried out on these 3 heavy metals. The rice plants were grown for 4 months in soil spiked with a mixture of three metals (Ni, Cr, and Fe), and their five-level of treatment of T1(25 mg/kg); T2(50mg/kg), T3(100mg/kg), T4(200mg/kg) and T5(400mg/kg). In the phytoremediation study, two types of control were used, TC1 (tap water) and TC2 (mimic control). Tap water control was

Table 2 General parameters of experimental soil

Parameter tested	Experimental Soil
pH	6.7±0.15
Color	Dark grey black yellow
Consistency	Less sticky
Sand (%)	43.3±2.2
Slit (%)	35.33±0.5
Clay (%)	17.72±0.2
Organic matter (%)	1.52±0.48
EC (dS m^{-1})	0.53±0.01
Nitrogen (kg/hectare)	189.14±8
Phosphorus (kg/hectare)	10.37±0.64
Potassium ((kg/hectare)	170.43±5
Sulphur (mg/kg)	10±0.4
Metals (mg/kg)	
Cd	BDL
Cr	0.091±0.06
Cu	1.40±0.3
Zn	0.36±0.5
Fe	4.64±0.1
Ni	0.28±0.15
Mn	3.12±0.2
Pb	BDL

BDL: below detection limit

Table 3 Root and Shoot length of rice plant treated with different concentrations of PTHMs after maturation

Treatments	Root Length (cm)	Shoot Length (cm)
TC1 (Tap water)	18.9±1.05 [a]	64.5±6.57 [a]
TC2 (Mimic control)	22.0±1.5 [b]	68.0±2.89 [b]
T1	22.8±3.16 [b]	71.0±8.6[b]
T2	24.1±1.1 [c]	72.5±2.79 [b]
T3	23.02±1.16[b,c]	71.5±3.9 [b]
T4	25.6±1.94 [d]	76.0±3.65 [c]
T5	21.3±1.08 [b]	69.0±3.45[a,b]

Different letters up to down in separate column shows significant variation (p=<0.05).

used to see the effectiveness of the process of phytoremediation, while mimic control (same as a combination of metals found in tannery wastewater) was used to evaluate the effects of the heavy metal status of the wastewater on rice plant.

The root and shoot lengths were significantly high in TC2 (mimic control) as compared to normal control (TC1). No significant differences were reported in the mean value of plant root and shoot lengths, and among all treatments, the highest root and shoot lengths were recorded in the T4 treatment (25.6 and 76.0 cm respectively) while the lowest root and shoot lengths were recorded from the treatment T5 (21.3 and 69.0 cm respectively). These results suggested that higher concentrations of heavy metals significantly inhibit the root and shoot length of rice plants (Table 3). Further, the effect of metal concentration on the fresh as well as dry weight of the rice plant was also evaluated and presented in

Table 4 Distribution of fresh and dry weight of rice plant

Treatments	Fresh Weight in Gram				Dry Weight in Gram			
	Root	Shoot	Penicles	TW	Root	Shoot	Penicles	TW
TC1	1.45	8.90	1.077	11.44[a]±4.46	0.84	3.366	0.538	4.74[a] ±4.52
TC2	4.45	16.8	1.797	23.1[b] ± 14.8	3.099	7.843	1.526	12.47[b] ±5.88
T1	4.10	19.6	1.053	24.8[b] ±10.98	2.684	8.054	1.435	12.17[b] ±6.42
T2	4.92	27.3	2.015	34.32[c] ±9.84	2.87	9.317	0.833	13.02[b] ±4.93
T3	5.12	21.1	0.479	26.77[b] ±6.89	3.393	8.121	0.26	11.7[b] ± 7.83
T4	6.12	32.9	2.606	41.7[d] ± 6.82	3.074	10.898	1.602	15.57[b] ±7.65
T5	5.95	22.1	1.004	29.1[b,c] ±6.15	4.119	8.663	0.574	13.3[b] ±4.05

TW: total weight of plant;Different letters shows significant variation and same letter shows homogeneity of the data set in separate column

Table 4. Compare to plants grown in unspiked soil (controls), treatment T1 (25mg/kg) to T5 (400mg/kg) represent an increment in fresh weight ($p<0.05$). The mean value of dry weight is increasing with increasing metal concentration but this difference was found non-significant ($p> 0.05$).

3.4 Effect of metal concentration on biochemical parameters

Under PTHMs stress, plants stimulate redundant reactive oxygen species (ROS) which commences oxidation harm such as oxidation of proteins and lipid peroxidation, inhibition of enzymes and genetic materials. No significant changes were observed in 1st month of treatment. As compared to the plants grown in unspiked soil/uncontaminated soil (control) plants grown in PTHMs contaminated soil (T1 to T5) have higher oxidative stress (Figure 1). The highest MDA level (nm/gm) was recorded from the T5 treatment pot as compared to its lower level and control after 2.5 and 4th months. The raised MDA content suggested that intracellular generation of free radicals leads to membrane damage and different cytotoxicological effect by polyunsaturated lipid generating MDA as one of the by-products of lipid peroxidation. In plants, high lipid peroxidation blocks the flow of electrons in photosystem II by metallic ions which generates chlorophyll and thereby generates free radicals (Khorobrykh et al. 2020). In addition, fatty acid supports the structure of harmful lipid peroxides that develop MDA in plants (Sidhu et al. 2016). The accrual of MDA at a high rate in plant tissue usually reflects drastic lipid peroxidation. Therefore, this progression of MDA also supports oxidative stress by modulating several biochemical and morphological attributes (Singh et al. 2015; Qamer et al. 2021). The results of the current study are in agreement with the findings of Singh et al. (2022) who have reported that excessive accrual of metals induces MDA levels in different parts of plants. Another study, done by Bharagava et al. (2008) also made similar conclusions and showed an elevated level of MDA in leaves of the mustard plant treated with different metals concentrations.

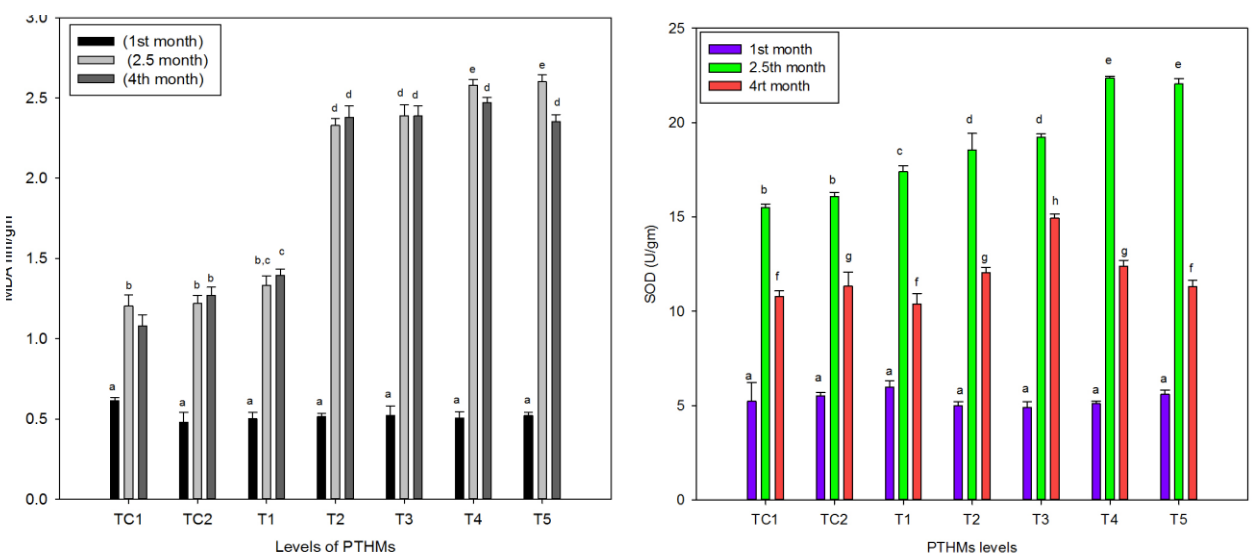

Figure 1 MDA and SOD level in the leaf of rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at $p<0.05$).

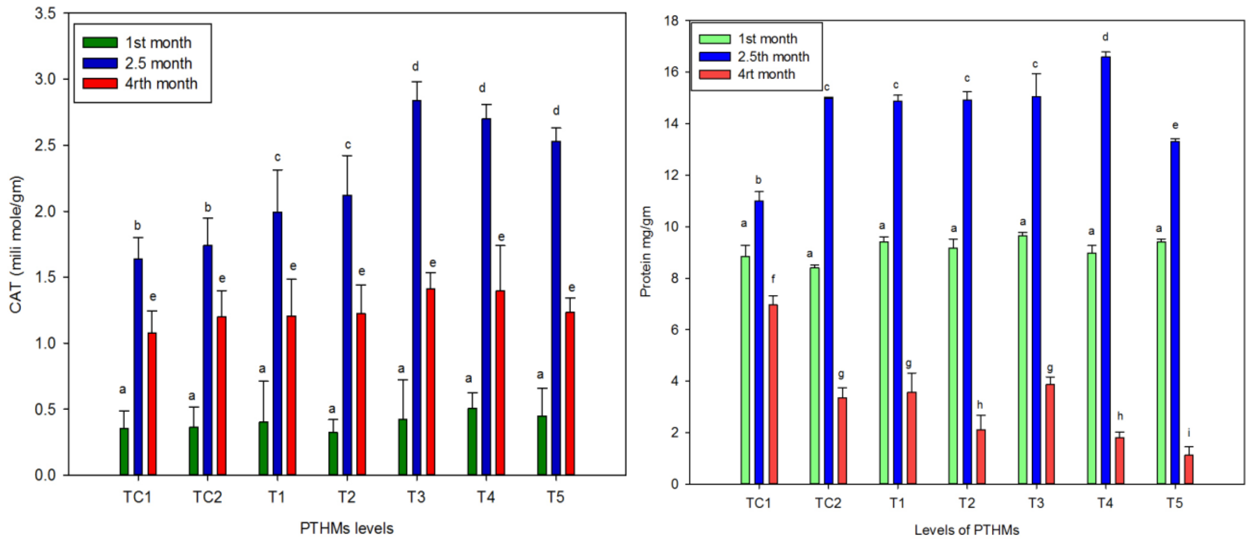

Figure 2 Distribution of Catalase (CAT) and protein level in the leaf of rice irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05).

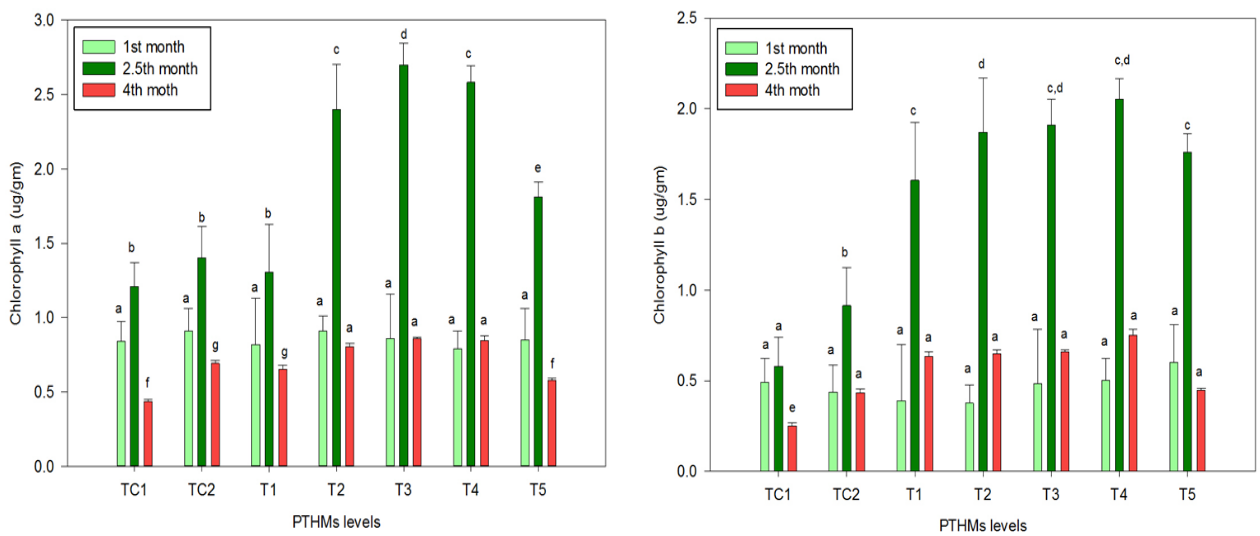

Figure 3 Distribution of Chlorophyll a, b in the Leaf of rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05).

The defense machinery of plants such as SOD (U/gm) and CAT (millimole/gm) levels were increased significantly with an increase in the concentration of heavy metals T1 (25mg/kg) to T4 (200mg/kg) than control (p<0.05). The maximum increase in SOD (U/gm) was recorded as 23.68±1.73 at T4 (200 mg/kg) treatment after 2.5month of growth period compare to lower level and control. Further, the SOD level was reduced after the 4th month (p<0.05). The Maximum CAT activity was found in plants treated with T3 (100mg/kg) of PTHMs (2.72±0.43 millimole/gm). Whereas, when the concentration increases above T4 (200 mg/kg) the level of antioxidants (SOD and CAT) was reduced. At the end of the 4th month, the enzymatic activities were reduced significantly as compared to 2.5 months (p<0.05). Several independent studies suggest that under sustained environmental stress, the battle between oxidative stress and their scavenging machinery is imbalanced and causes increased oxidative stress which may trigger different types of harmful effects on plants (Farooq et al. 2016; Khorobrykh et al. 2020). The content of protein in the plant leaves was detected higher during the entire growth period as compared to the controls. Like the other two parameters, in the case of protein also, maximum protein content was detected in T4 (200mg/kg), and hereafter this, when the concentration of PTHMs increases the level of protein was decreased gradually (Figure 2).

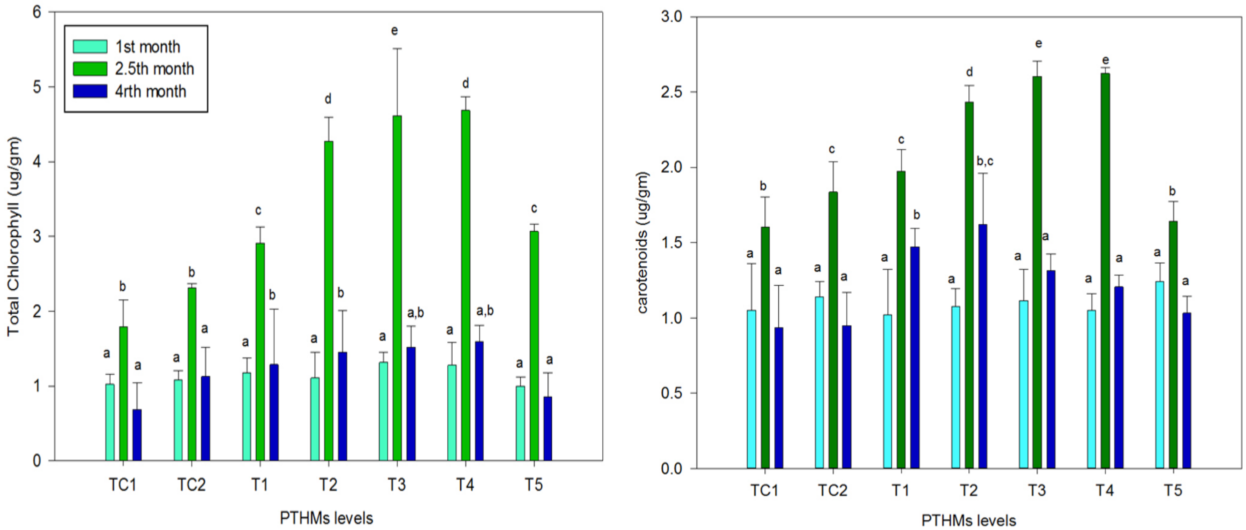

Figure 4 Distribution of total Chlorophyll and carotenoids in rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05)

The maximum elevations of all chlorophyll content were recorded after 2.5 months of the growth period. The concentration of these pigments reduced drastically after 4 months as compared to 2.5 months. The maximum increase in total chlorophyll, chlorophyll a, b, and carotenoid level were found at 2.5 months in treatment T4 (200 mg/kg) (Figures 3 and 4). This rise in chlorophyll levels in the leaves of the rice plant may be due to the presence of high Cr, Fe, Ni, etc. These findings are in agreement with the earlier results reported (Farooq et al. 2016; Amari et al. 2017; Singh et al. 2022). Moreover, a reduction was observed in chlorophyll and carotenoid level after the T4 (200mg/kg) PHTMs treatment levels ($p<0.05$). This change may be due to the interference of metals in chlorophyll biosynthesis through direct inhibition of enzymatic steps. For example, high Cr is well reported to hinder the biosynthesis of chlorophyll and decrease total chlorophyll as well as chlorophyll a/b ratio (Singh et al. 2022).

3.5 Accumulation and distribution of heavy metals in plant parts

The mean values of metal accumulation in different parts of rice plants irrigated with different concentrations of Cr, Fe, and Ni with the statistical measures are presented in Table 5. Different type of crop plant works differently for the accrual and dispersal of metals in their plant parts (roots, shoots, fruits, or seeds). Results revealed that this rice plant has shown maximum accrual of Fe (330.2±0.7

Table 5 A Comparative accumulation pattern of metals (mg/kg) in different parts of Rice plant irrigated with different concentration of metals.

Metals	TC1 (Normal control)	TC2 (TWW)	T1 (25)	T2 (50)	T3 (100)	T4 (200)	T5 (400)
Chromium							
Root	BDL	7.77^{a}±0.08	2.23^{a} ±0.1	13.34^{a} ±0.04	34.68^{a} ±0.57	41.96^{a} ±0.05	46.69^{a} ±0.1
Shoot	BDL	1.30^{a} ±0.02	0.84^{a} ±0.1	9.88^{a} ±0.05	28.46^{a} ±0.05	37.87^{a} ±0.05	40.78^{a} ±0.8
Seed	BDL	BDL	BDL	0.01^{a} ±0.01	0.02^{a} ±0.02	1.07^{a} ±0.05	1.12^{a} ±0.07
Nickel							
Root	1.8^{a} ±0.1	2.16^{a} ±0.04	10.01^{a} ±0.04	16.67^{a} ±0.6	21.21^{a} ±0.42	26.34^{a} ±0.81	30.22^{a} ±0.7
Shoot	1.1^{a} ±0.1	1.65^{a} ±0.07	7.09^{a} ±0.01	14.67^{a} ±0.8	18.89^{a} ±0.76	24.44^{a} ±0.58	26.0^{a} ±0.76
Seed	BDL	0.62^{a} ±0.08	1.29^{a} ±0.071	1.43^{a} ±0.05	1.72^{a} ±0.011	2.06^{a} ±0.014	2.70^{a} ±0.02
Iron							
Root	3.37^{a} ±0.05	6.07^{a} ±0.52	30.07^{a} ±1.6	79.95^{a} ±4.5	160.24^{a} ±12	222.25^{a} ±13	330.2^{a} ±19
Shoot	1.50^{a} ±0.1	3.85^{a} ±0.01	20.17^{a} ±0.05	68.6^{a} ±1.82	141.13^{a} ±8.1	202.9^{a} ±10.5	277.7^{a} ±12
Seed	0.8^{a} ±0.1	2.75^{a} ±0.05	5.69^{a} ±0.85	12.28^{a}±1.4	23.33^{a} ±1.75	46.38^{a} ±1.71	57.91^{a} ±2.8

Different letters in a row show significant variation and same letter shows homogeneity of the data set mg/kg) followed by Cr (46.6±0.1 mg/kg) and Ni (30.22±0.7 mg/kg) in roots. The accrual of all tested heavy metals was found maximum in roots followed by shoot and/or seeds, which increased with increasing concentration of metals T1 (25mg/kg) to T5 (400mg/kg). The reason for the high accrual of heavy metals in roots may be due to the high metabolic rate of other plant parts (Phusantisampan et al. 2016; Singh et al. 2021).

This study revealed that concentrations of all heavy metals in seeds of rice plants were found below as recommended by different environmental agencies up to T4 treatment (200 mg/kg) PTHMs. But, as the concentration of heavy metals increased above T4 (200 mg/kg) and onwards, metals concentration in seeds of rice plant were increased and exceeded the permissible limit i.e. 0.02 mg/kg, 1.63 mg/kg, 20.0 mg/kg for Cr, Ni, and Fe respectively for human consumption (Bhargava et al. 2008). Therefore, it may be advisable that the rice plants treated with a high concentration of heavy metals (above 200 mg/kg) are not suitable for human and cattle consumption (Ahmad et al. 2022).

3.6 Translocation factors

According to Baker and Walker (1990), plants are categorized into three groups as per their strategies for growing on metal-polluted soils; indicators, accumulators or hyperaccumulators, and metal excluders. The efficacy of plants for the absorption and distribution of metals is judged based on the bioaccumulation factor (BAC) and translocation factor (TF). Results showed that experimental plants growing at different PTHMs levels have the potential to accumulate metals.

In this study, metal concentrations in the potting soil were taken up to 400 mg/kg (T5) and the maximum accumulation by roots and shoot of rice plant was 330.17±19.56 for iron. Hence, the calculated bioaccumulation factor was less than <1 (Yan et al. 2020). Hence, we can state that rice plants can effectively limit the heavy metal translocation within them and maintain relatively low levels of metals in their shoot over the huge amount present in the soil. Further, metal distribution in different parts of the rice plant (root to shoot and shoot to seed) was also evaluated based on the translocation factor (TF). TF is a relative translocation of metals from root to shoot or shoot to seed in plants. The TF for root to shoot was gradually increased as the concentration of Cr, Fe and Ni increased (Figure 5). The maximum TF (Cr: 0.9, Fe: 0.88 and Ni: 0.89) was recorded in the 200 mg/kg (T4) PTHMs treatment whereas minimum TF was found in the control (TC1=Cr: 0.0; Fe: 0.43 and Ni: 0.6). Further, this pattern was also followed by the translocation factor from shoot to seed. These results were

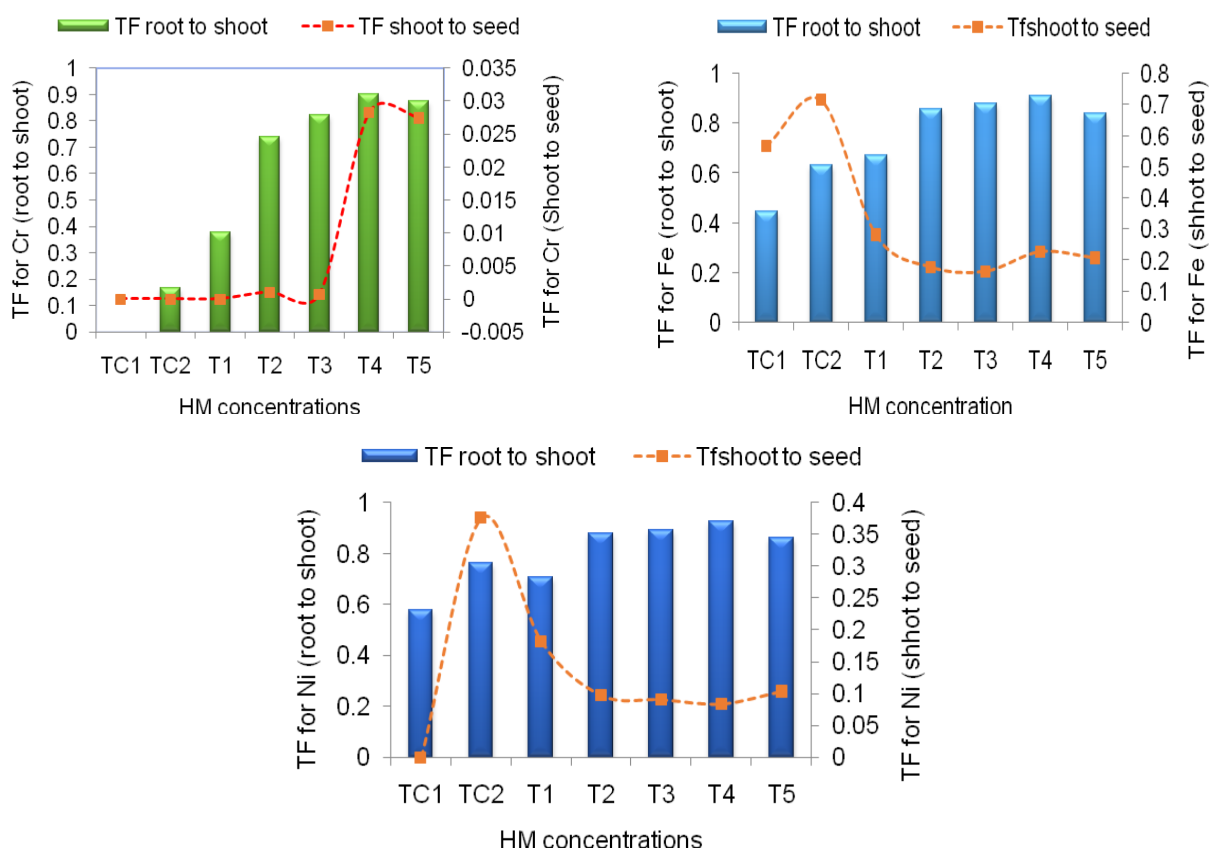

Figure 5 Distribution of metals on the basis of translocation factor (TF) from root to shoot and shoot to seed at different treatment

consistent with Alrawiq et al. (2014) and Kahangwa et al. (2021). The TF for root to shoot was increased as the concentration of metals increased (Figure 5). Plants generally not accumulated those heavy metals that are not needed in metabolic use. The translocation of toxic metals (Cr, Ni) may be restricted by roots to other organs due to blockage by casparian strips in the endodermis. These results were corroborated with the findings of Jamla et al. (2021).

Conclusion

Significantly higher accrual of Cr, Fe, and Ni were observed in different parts of the rice plant treated with different levels of heavy metals. This accrual may directly or indirectly impede various metabolic and biochemical activities along with oxidative damage by altering the enzymatic structure and their regulation. Accumulation and TF of heavy metals suggested that it is effective in removing PTHMs up to 3 times higher than the level of heavy metals present in TWW at present. Therefore, it can be used for the management of multi-metal polluted wastewater such as tannery wastewater or other wastewater but at certain limits (not more than 200 mg/kg). If it is treated above this, then seeds were also affected and not safe for human consumption. Further, the study revealed that plants irrigated with above T4 (200mg/kg) concentration have accumulated toxic metals in seeds beyond the permissible limit as suggested by different environmental agencies for human consumption.

Author contribution

ASK performed, analyze and drafted the manuscript, ASK, MB, DS, AA and AS participated in the experimental design and performed GC-MS and ICP-MS analysis, DS, AS, AA and VG conceptualized and finalize the manuscript. All authors approved the final manuscript.

Acknowledgment

We thank M/s Unnao tannery pollution control company, A-7, Site-II, UPSIDC, industrial area. Unnao, 209101, U.P., India for providing the tannery effluent samples.

Conflict of interest

None

Financial support and sponsorship

Nil

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