EVALUATION OF THE POTENTIALITY OF *SESBANIA CANNABINA* (RETZ.) POIR. FOR PHYTOREMEDIATION OF HEXAVALENT CHROMIUM CONTAMINATED SOIL.

By
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Abstract

Heavy metals (HM) accumulation in the soil or sediment is of significant environmental concern because of their toxicity to living organisms. Hexavalent chromium [Cr(VI)] is considered one of the most toxic HM released in the natural environment due to anthropogenic activities and they are still discharged from many industrial processes (e.g., chromo-tanning in leather industries), especially in developing countries like Bangladesh due to continuing use of chromium-containing salts (e.g. Na_2CrO_4, $K_2Cr_2O_7$, $Cr(OH)SO_4$). These HM do not decompose or disintegrate thus, removing or converting them into a less harmful state will reduce bioavailability and toxicity. Using plants (phytoremediation) in the remediation of HM has many advantages over chemical methods because it is less costly and can change metal speciation without the addition of further potential contaminants. The prerequisite for being a phytoremediation species is that species need to be fast-growing, able to grow under broad environmental stress and non-edible. A native species in Bangladesh *Sesbania cannabina* (Retz.) Poir. fulfils these conditions. This research aims to assess the Cr(VI) phytoremediation capacity of *Sesbania cannabina* (Retz.) Poir. (commonly known as Dhaincha in Bangladesh).

Study 1 (Chapter 2) provides evidence of Cr(VI) contamination in riverbank sediment (the highest Cr(VI) was recorded at 31.67±2.87 ppm, December 2021) of the Dhaleshwari River, Bangladesh. In Study 2 (chapter 3), we optimised the conditions (seeds pre-treated with H_2O_2 (6% v/v) for 5 minutes and primed with 65°C water for 5 minutes can germinate well at 27.5 ± 2.5°C) for *S. cannabina* seed germination. In study 3 (chapter 4), we tested the effect of Cr(VI) concentration on seed germination and root radicle elongation, and we observed *S. cannabina* can germinate and grow in

concentrations of up to 175 ppm Cr(VI) in a growth medium. In addition, we observed the effect of Cr(VI) on the root system of *S. cannabina* by using rhizobox in study 4 (chapter 5) and found that root growth in plants grown in 160 ppm contaminated soil was reduced by about 55±0.65% at 25 days and 35±0.25 % at 45 days and that the root system was destroyed ≥360 ppm. Finally, we studied (chapter 6) the phytoremediation (in soil) potential of *S. cannabina* under different concentrations of Cr(VI) and observed that the plant species can convert all the harmful Cr(VI) to less harmful Cr(III) when grown in ≤ 175 ppm Cr(VI) contaminated soil.

Collectively, the four studies mentioned above (chapters 2 to 5) suggest that *S. cannabina* is a suitable candidate for phytoremediation of Cr(VI) (≤ 175 ppm) contaminated soil because it can convert all Cr(VI) to Cr(III) and sequester chromium in the roots.

DEDICATION

TO MY **CREATOR** AND MY **FAMILY**

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LIST OF PUBLISHED ABSTRACTS

This thesis incorporates the following abstract:

- Abdul Kadir Ibne Kamal, Lesley Batty, and Rebecca Bartlett; “Evaluation of the root system and phyto-management potential of Sesbania cannabina grown in hexavalent chromium contaminated soils utilizing modified rhizobox systems.” EGU General Assembly 2022; EGU22-4921; https://doi.org/10.5194/egusphere-egu22-4921.

- Abdul Kadir Ibne Kamal, Lesley Batty, Rebecca Bartlett, Suleiman Suleiman, and Joanna Chustecki; “Germination potential of Sesbania cannabina in 2 Chromium (Cr) spiked growth media” EGU General Assembly 2020; EGU2020-5952; https://doi.org/10.5194/egusphere-egu2020-5952.

- Abdul Kadir Ibne Kamal, Lesley Batty, and Rebecca Bartlett; “Assessing the Buriganga River Pollution of Bangladesh: Past, Present and Future.” *Research poster conference*, University of Birmingham, 19th June 2019. https://intranet.birmingham.ac.uk/as/studentservices/graduateschool/documents/public/RPC-2019/RPC-2019-Conf-Booklet.pdf

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Paper 2:

Title : **Optimisation of seed germination and seedling emergence of *Sesbania cannabina* (Retz.) Poir.**

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Paper 3:

Title : **Effect of chromium on seed germination and root development of Sesbania cannabina (Retz.) Poir. in two different growth media.**

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Paper 4:

Title : **Evaluation of the root system of *Sesbania cannabina* (Retz.) Poir. grown in hexavalent chromium contaminated soils by utilising modified rhizobox systems.**

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Paper 5:

Title : **An assessment of the phytoremediation potential of *Sesbania cannabina* (Retz.) Poir. grown in hexavalent chromium contaminated soil.**

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Chapter 1:

General Introduction and Literature Review.

1.1 Prelude

Worldwide, soil contamination with heavy metals (HM) has become a global public health issue for the safety concern of agricultural products and food safety (Li et al., 2019; Vardhan et al., 2019). Globally a significant area of land (about 20 million ha of land) of > 500 million sites are contaminated with heavy metals or metalloids, and the concentration of these contaminants is above the standard or regulatory levels (Liu et al., 2018; He et al., 2015). The monetary consequences of this soil pollution are estimated to be >US$10 billion per year and hinder future economic growth (He et al., 2015). At present major polluting industries are located in developing countries, and these industries directly or indirectly pollute the soil and rivers of these countries. Urban rivers and sediments of developing countries are also contaminated with heavy metals, mostly because sewage and industrial effluent are discharged directly into the river channel without proper treatment (Tom et al., 2014).

Bangladesh is a developing country; its capital, Dhaka, is surrounded by three rivers, the Buriganga, Turag and Shitalakhya. Along with economic growth (GDP growth of 8.2% in 2019), rapid population growth (more than 21 million people in 2020) and improper management of wastes (including wastewater from industries) in Dhaka increase the pollution load in the surrounding rivers of the capital (WPR, 2020; World Bank, 2020). Major polluting industries such as tanneries, textile industries and battery recycling are situated on the bank of these rivers and discharge effluents directly into the river without appropriate management (Islam et al., 2018a; Nargis et al., 2018; Islam et al., 2015). Heavy metals (such as Cr, Pb, Cd, etc.) are considered the most toxic substance among the major environmental pollutants (Liu et al., 2018; He et al., 2015).

Chromium (Cr) is recognised as a heavily noxious HM but is extensively used in processing industries (Shahid et al., 2017; Sinha et al., 2018; Jeřábková et al., 2018). It is in group VI-B transitional element (electronic configuration of Ar $3d^5 4s^1$) and has two steady oxidation states [Cr(III) or Cr^{3+} and Cr(VI) or Cr^{6+}] (Sueker, 2005). It can be found in all environment settings with various species (compounds) and different concentrations. Due to carcinogenicity and toxicity, compared with other chromium species, hexavalent chromium (Cr(VI)) is identified as a major environmental threat to both aquatic and terrestrial life (Jeřábková et al., 2018; Zayed and Terry, 2003).

Governments and the scientific community are trying to remediate the heavy metal-contaminated soil by several methods (Vardhan et al., 2019; Liu et al., 2018). Among them, phytoremediation is considered cost-effective and more sustainable but is still not highly efficient (Ashraf et al., 2019; Grzegórska et al., 2020). In this chapter, we will discuss the pollution status of river bank sediment of one of the major rivers in Bangladesh, the toxicity and chemistry of chromium and the existing phytoremediation methods.

1.2 Buriganga and Dhaleshwari River

1.2.1 River System

The Dhaleshwari-Buriganga system started near the meeting point of the Padma (Ganges) and upper Meghna rivers in Bangladesh (Point A in figure 1.1) (Kamal et al., 1999; Khan, 2004). The Dhaleshwari River is a tributary of the Buriganga river , and the Dhaleshwari River starts from the Meghna River, just upstream of the Padma (Ganges) river near the confluence (Point B in figure 1.1). But the Turag River is the primary source of water for the Buriganga, which receives flows from

local rainfall and the Jamuna River. The lower reaches of the Dhaleshwari-Buriganga system are tidal during the dry season when upstream inflows are minimal (Kamal et al., 1999; Khan, 2004).

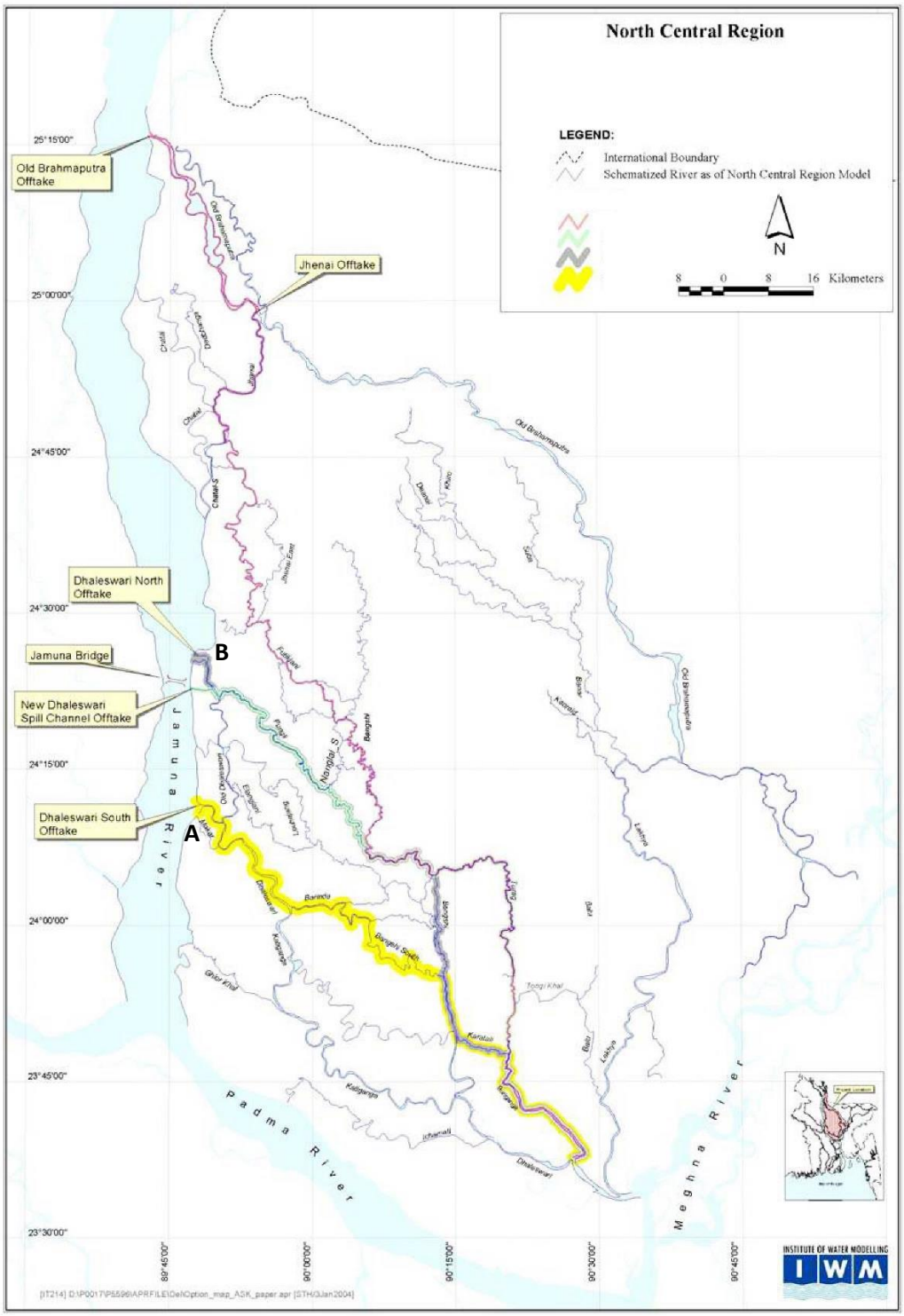

Figure 1.1: Buriganga- River system (modified after Khan, 2004)

1.2.2 River pollution

The main source of water for Bangladeshi rivers is from upstream rivers generated from the Himalayan mountain range of India; thus, the mean discharge rate varies from season to season. The water pollution also varies with the season, and during winter or dry season, these rivers become more polluted than the time of monsoon due to (dilution) heavy rainfall (Islam et al., 2015; Bhuiyan et al., 2015; Islam et al., 2014; Kolås et al., 2013).

The Department of Environment of the Bangladesh Government has detailed records on EC (Electric Conductivity) of Buriganga river water which is a useful index of water pollution parameters (DoE, 2014, 2015, 2017, 2013). The presence of mixed fish species in a river system is one of the key indicators of good water quality or good biological health of a river; mixed fishes can only survive in the EC range between 150 to 500 micro-siemens per centimetre (µs/cm) and EC value above it is not suitable for certain kind of fish or macroinvertebrates (EPA, 2012).

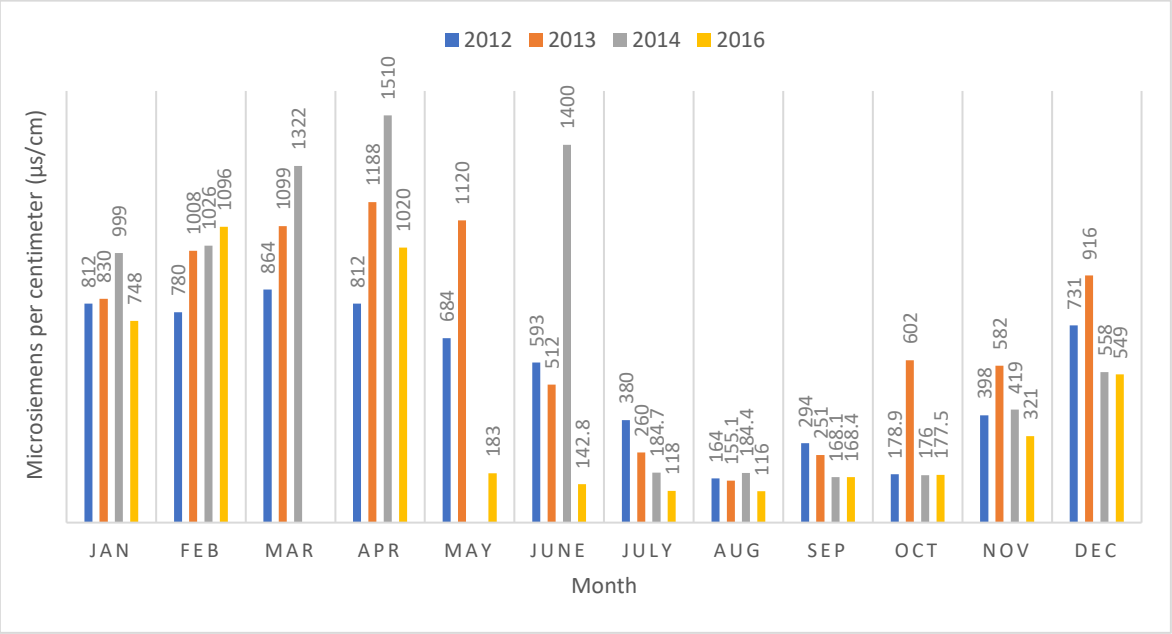

Figure 1.2: EC value at near Kamrangir Char of Buriganga River water in the year 2012, 2013, 2014 and 2016. (DoE, 2014, 2015, 2017, 2013).

River water near Kamrangir Char, Dhaka, of the mainstream of the Buriganga River was reported to be the most polluted site and figure 1.2 shows the EC value of that site (Islam et al., 2018b; Nargis et al., 2018). From the EC value, the highest contamination was detected in Feb-Mar-Apr (780 to 1510 µs/cm), when rainfall and water discharge from upstream is low. In Aug-Sep-Oct, contamination is lower due to dilution by heavy rain and high discharge from upstream.

Throughout the year, Biological oxygen demand (BOD) and Chemical oxygen demand (COD) were always above the threshold limit highest value observed BOD 284 ppm (BOD permissible limit in the freshwater ecosystem ≤ 5ppm)in December 2016 and COD was 212.6 ppm in December due to the discharge of untreated effluent from textile industries, tannery industries and sewage from Dhaka city in the river, which typically contains chemically reduced compounds, that readily react with oxygen (Bhuiyan et al., 2015; Islam et al., 2015; Saifullah et al., 2012; DoE, 2017, 2015)

1.3 Chromium in Environment and their effects

1.3.1 Sources of Chromium

Chromium is the 24th most abundant element in the earth's crust (average 100–200 ppm as total chromium), and ultramafic (basaltic igneous) rocks and their derived soils have higher chromium concentrations (Jeřábková et al., 2018; Becquer et al., 2003). South Africa, India and Kazakhstan are the leading countries for mining chromium as chromite (USGS, 2020; Shahid et al., 2017). Globally, chromium production by mining increased progressively between 2000 and 2019 from 14,400 thousand metric tons to 44,000 thousand metric tons, respectively (USGS, 2020) (figure 1.3).

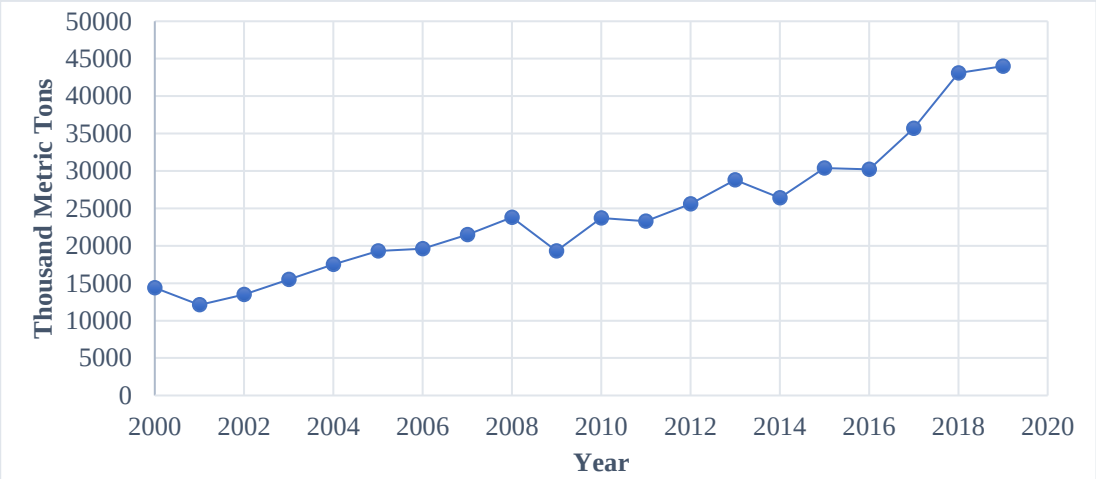

Figure 1.3: Annual world mine production of Cr (USGS, 2020).

Chromium compounds are used in metallurgical and galvanising, chromo paints and pigments, rawhide processing (tanning), furniture, pulp and paper industries. Stainless steel and alloy preparation industries consume 65.0 to 70.0 % of the total production of chromium (Sinha et al., 2018; USGS, 2020). More than 85% of the leather tanning industries use Cr salt as a tanning agent, and their effluents contain chromium (Sundaramoorthy et al., 2010). In most developing countries like Bangladesh, these industries discharge their chromium-containing effluent straight into the environment without proper treatment (Shahid et al., 2017; Jobby et al., 2018; Sinha et al., 2018; Jeřábková et al., 2018).

1.3.2 Geochemistry of Chromium

Geochemical processes govern the speciation, transport and fate of heavy metals in the environment. A wide range of chromium oxidation states have been found, varying from -2 to +6. Among these states, elemental chromium (Cr 0), trivalent chromium (Cr(III)), and hexavalent chromium (Cr(VI)) are the most stable (Sueker, 2005; Kim and Dixon, 2002). Pourbaix diagram (Eh-pH diagram) illustrates the

behaviour of chromium species under specific Eh (reduction-oxidation (redox) potential) and pH conditions (figure 1.4) (Sueker, 2005; Palmer and Wittbrodt, 1991).

Thermodynamic stability of chromium species also depends on the presence of atmospheric oxygen, pH and complexing agent (excluding H_2O or OH^-) (Bandara et al., 2020). According to the Pourbaix diagram of chromium, in high Eh and high pH, hexavalent chromium (i.e., $HCrO_4^-$ and CrO_4^{2-}) is thermodynamically steady. By contrast, in low pH and all Eh conditions, trivalent chromium (i.e., Cr^{3+}, $CrOH^{2+}$, $CrOH^{2+}$, $Cr(OH)_2^+$, $Cr(OH)_3^0$ and $Cr(OH)_4^-$) is thermodynamically steady (Bandara et al., 2020; Hu et al., 2016). In addition, under reducing conditions and high pH, trivalent chromium is also stable.

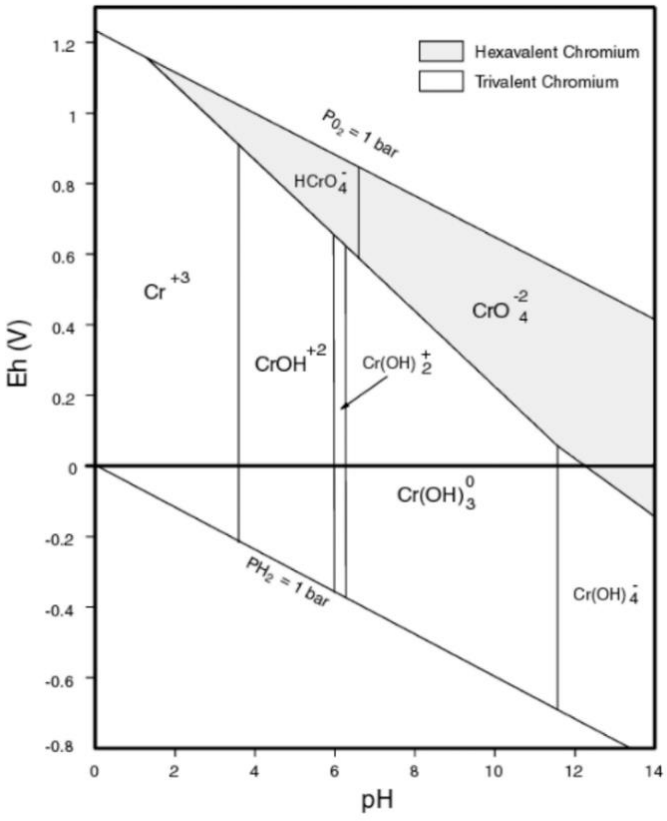

Figure 1.4: Eh-pH diagram for chromium (Sueker, 2005).

Eh-pH diagram shows distinct boundaries between Cr(III) and Cr(VI) species, but the mechanism of conversion from one species to another is unclear (Bandara et al., 2020; Hu et al., 2016). Cr(III) species are the most dominant species because they occur in wide-ranging Eh and pH values; below pH 3, they are predominant as ionic (i.e., Cr^{+3}). By contrast, Cr(VI) can be converted to Cr(III) under highly acidic conditions because, under acidic conditions, chromate acts as a potent oxidising agent (Fendorf et al., 2000; Kim and Dixon, 2002; Sueker, 2005).

1.3.3 Toxicity of chromium

Trivalent chromium (Cr(III)) can play important roles in maintaining normal glucose function (recommended value 125 to 600 µg/d) and lipid metabolism in humans (Paiva et al., 2015; Behrouz et al., 2020; Anderson, 2003). By contrast, hexavalent chromium (Cr(VI)) is highly toxic and carcinogenic in even low doses (Hu et al., 2018; Peralta-Videa et al., 2009). In summary, Cr(VI) is the most toxic species of chromium and chromium (III) species are less toxic and cause fewer health problems (Wilbur et al., 2012; ATSDR, 2012; Hu et al., 2018; Browning and Wise, 2017).

Chromium can enter the human body through inhalation and oral routes (ATSDR, 2012). Low doses (0.003 ppm/day) of Cr(VI) can cause iron deficiency (anaemia), allergic reactions in the body, skin irritations, gastric ulcers and infertility in men (ATSDR, 2012; Wilbur et al., 2012). High doses (> 250 ppm) can cause severe coronary heart disease, asthma, gastrointestinal and hepatic disorders, and damage to the mitochondria and DNA of blood cells, leading to carcinogenicity (ATSDR, 2012; Ray, 2016; Hu et al., 2018). Alterations in cellular DNA have been observed in many i*n vivo* and *in vitro* experiments when exposed to chromate

compounds (Hu et al., 2018; Browning and Wise, 2017), and the link between C(VI) and carcinogenesis in human cells has been clearly demonstrated (Browning and Wise, 2017).

Chromium is not recognised as an essential nutrient for plant development (Zayed and Terry, 2003; Ertani et al., 2017; Stambulska et al., 2018). Although plants take up Cr(VI) actively like other essential ions, the uptake mechanism of Cr(III) is passive (Pradas del Real et al., 2020; Peralta-Videa et al., 2009; Shahid et al., 2017). However, chromium toxicity in plants depends on the composition, concentration and distribution of different chromium species in soil and Cr(VI) perform higher toxicity on plants rather than Cr(III) species (Zayed and Terry, 2003; Hayat et al., 2012; Shahid et al., 2017). Studies on several crop plants have shown chromium disruption of photosynthesis activities by inhibiting electron transport, disrupting the activation of Calvin cycle enzymes and decreasing CO_2 fixation (Panda and Choudhury, 2005; Rocchetta and Küpper, 2009). Plants have also shown reduced water potential, elevated transpiration rate, wilting and changes in the tracheary vessel when chromium is taken up from contaminated soil (Singh et al., 2013; Shanker et al., 2005). Several studies of chromium toxicity on soybean, tomato, bush bean, sunflower, and maise plants showed that the uptake of essential elements (Mg, Fe, Ca and P) was hindered by chromium (Panda and Choudhury, 2005; Singh et al., 2013; Shanker et al., 2005).

Vegetables satisfy the major portion of the human diet, prevent diseases and are sources of major vitamins (NHS, 2018; Siegel et al., 2014; Dias and Ryder, 2011). Farmers in developing countries often irrigate their land with polluted river water (Qadir et al., 2010; Khan et al., 2008) and also grow vegetables on landfill sites for urban markets (Kamal et al., 2016). Plants can accumulate HMs from aqueous

medium and soil and in this way, heavy metals enter the food chain (Zwolak et al., 2019; Oves et al., 2012). Therefore soil and water contaminated with chromium become a major concern in food production because crop plants can and do accumulate chromium while cultivated in contaminated environments (Chen et al., 2014; Ertani et al., 2017; Khan et al., 2008).

1.3.4 Soil and Sediment Guidelines for Chromium

Traditionally arid and fertile soil has been used in agricultural practices, and generally, this soil contains low levels of HM suitable for agriculture (Ertani et al., 2017). Natural levels of HMs in soil and sediment are altered because of industrial activities and the application of excessive fertiliser/ soil amendment (He et al., 2015; Oves et al., 2012).

These elevated levels of heavy metals affect living organisms, but each heavy metal's threshold level varies between the individual element and species (ionic state)(Vardhan et al., 2019; Oves et al., 2012). Soil texture, organic matter and pH strongly affect the mobility and bioavailability of HMs (Oves et al., 2012; Sinha et al., 2018; ATSDR, 2012).

Soil quality is vital for land management in rural and urban environments. As heavy metal contamination is not only a regional problem, combined global efforts by governments, scientists and communities are required to control and mitigate the problem (Liu et al., 2018; Vardhan et al., 2019). For the purpose of controlling and mitigation of HM pollution, a common regulatory standard is required for agricultural soil and sediments.

Table 1.1: Soil and sediment chromium standards (ppm dry weight).

Agency/ Guideline /Report	Chromium Species		Sediment (ppm dw)		Soil ppm dw	References
			Fresh water	Marine/ estuarine		
Canadian Sediment Quality Guidelines	Total Chromium	ISQGs	37.3	52.3		(CCME, 1999; MoE, 2004)
		PLEs	90	160	67	
	Cr(VI)	TV			2.5	
Ministry of the Environment, Finland	Total Chromium	TV			100	(MEF, 2007; Tóth et al., 2016)
		LGV	-		200 (e)	
		HGV			400 (e)	
Department of Environment and Conservation, The Government of Western Australia	Cr(VI)	EIL			1	(DoEC, 2010)
	Cr(III)	EIL			400	
	Total Chromium	ISQGs Low	80			
		ISQGs high	370			
Contaminated Land Exposure Assessment, Environment Agency, UK	Cr(VI)	Residential with home-grown produce			21	(ALS Environment, 2009)
		Residential without home-grown produce			21	
		Commercial			49	
	Total Chromium	Residential with plant uptake			130	
		Residential without plant uptake			200	
		Commercial and Industrial			5000	
United States Environmental Protection Agency	Cr(VI)	TV			11	(USEPA, 2004)
	Total Chromium	Soil level requiring clean-up			230	
		BERL	81			(NOAA, 1999)
		BERM	370			

Please note: Interim sediment quality guidelines (ISQGs); Probable effect levels (PELs); Threshold value (TV); Lower guideline value (LGV); Higher guideline value (HGV); Ecological risks (e); Ecological Investigation Levels (EIL); Biological Effects Range-Low (BERL); Biological Effects Range-Median (BERM).

However, different countries set their own critical / threshold value for each contaminant on the basis of exposure, local geography, land use and HM distribution (Table 1.1).

1.4 Remediation of heavy metals from contaminated sites

Methods used in the remediation of heavy metals from contaminated sites (soil) can be categorised as physical, chemical (Peng and Guo, 2020), biological (Grzegórska et al., 2020) or a combination of these techniques. Physical (e.g. soil vacuum and soil washing) and chemical (e.g. oxidation, neutralisation, and soil flushing) techniques alter the soil composition and texture and reduce the biological activities within the soil. By contrast, biological remediation processes reduce the risk associated with contamination whilst not negatively affecting overall soil quality using 'natural' biological attenuation. Soil remediation techniques can also be classified on the basis of remediation sites (e.g. ex-situ techniques and in-situ techniques). In ex-situ methods, polluted soil or sediments are transferred to other sites for decontamination of the contaminated soil or landfill, and this is one of the main causes of high remediation costs (Peng and Guo, 2020; Malaviya and Singh, 2011). By contrast, in-situ methods, for example, phytoremediation, allow decontamination to take place in on-site without excavation or translocation of the contaminated soil and sediment. In phytoremediation (in-situ method) plants act as bio-reactors in the remediation process and thus make it cheaper (Grzegórska et al., 2020; Igiri et al., 2018).

1.4.1 Physico-chemical methods of Cr removal

Precipitation, electrochemical, ion exchange, reverse osmosis and adsorption are well-known methods of the physico-chemical treatment process for the remediation of chromium, which is rapid and do not depend upon the weather, but these are not economically viable and generate noxious sludge (Peng and Guo, 2020; Pakade et al., 2019; Nur-E-Alam et al., 2020; Malaviya and Singh, 2011). Moreover, these

methods initiate more pollution load (total dissolved solids and conductivity) as secondary contamination from treated effluents and also affect soil fertility by destroying the biotic component (Sinha et al., 2018; Li et al., 2019).

1.4.2 Biological remediation

In biological remediation, microorganisms or plants are used to remove xenobiotic compounds (organic and inorganic) from the environment; the ultimate objectives of biological remediation are to restore the contaminated environment efficiently in a sustainable and economically viable way (Ashraf et al., 2019; Grzegórska et al., 2020). Various biological remediation methods (figure 1.5) have been introduced during the last two decades (Azubuike et al., 2016; Grzegórska et al., 2020). Much research has focussed on developing different biological remediation techniques; however, no single treatment method has yet been developed to decontaminate the environment because of various pollutants (Ashraf et al., 2019; Grzegórska et al., 2020).

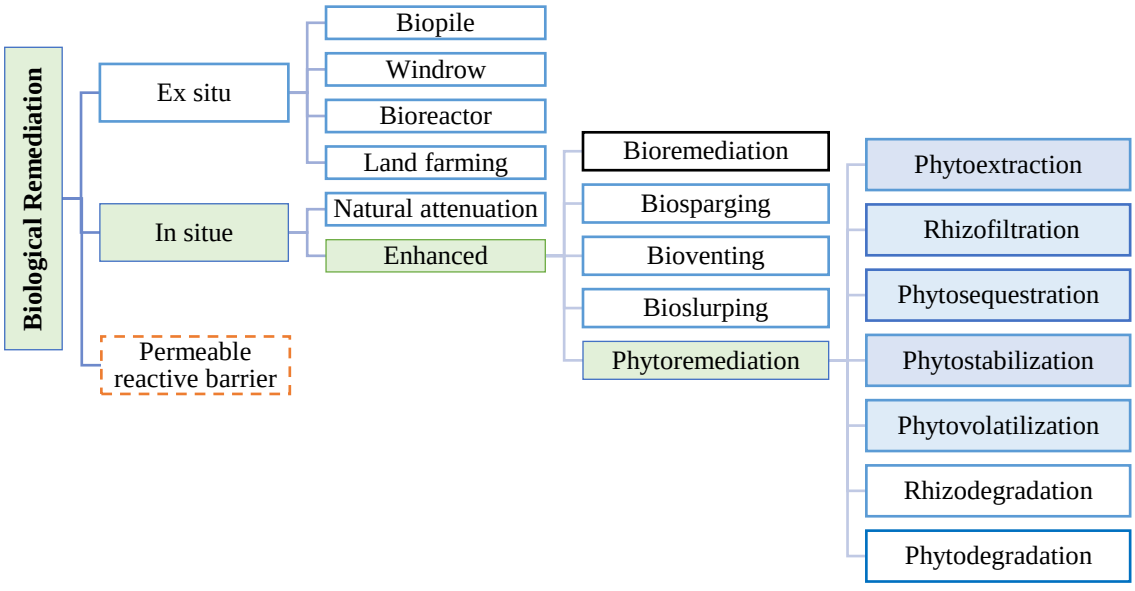

Figure 1.5: Biological remediation methods. Modified from Azubuike et al., (2016) and Grzegórska et al., (2020).

1.4.2.1 Phytoremediation of heavy metals

The process or technology related to the removal or reduction in the toxicity of contaminants into harmless substances by utilising plants and their associated microorganisms is known as phytoremediation (Grzegórska et al., 2020; Ashraf et al., 2019; Marques et al., 2009). Organic contaminants are usually biodegradable and can be successfully attenuated by phytoremediation, but biodegradation (phytodegradation and rhizodegradation) of HM is not achievable. Thus, phytoremediation of HM can be divided into five main subcategories (Figure 06), which may function simultaneously in phytoremediation (Ranieri et al., 2020; Sinha et al., 2018); these are :

(i) Phytoextraction removes the HM and radionuclides from the contaminated environment by accumulation in plant tissue (mostly in aerial parts) (Grzegórska et al., 2020).

(ii) Rhizofiltration is the adsorption or deposition of pollutants onto plant roots; biotic or abiotic processes are involved in this process (USEPA, 2000).

(iii) Phytosequestration: If heavy metals form complexation, precipitate or immobilise in the root and accumulate in the vacuole in the root cell; this process is known as phytosequestration (Ali et al., 2013; Ahmad et al., 2011).

(iv) Phytostabilisation occurs when plant roots immobilise (onto roots) and precipitate (in rhizosphere) metals in soil or sediments (Wu et al., 2010).

(v) Phytovolatilisation is limited to very few elements, such as Hg and Se, and occurs when plant leaves transform noxious substances into atmospheric volatiles. (Ali et al., 2013).

However, different Plant species react differently to individual HM and on the basis of their accumulation of HM plants can be categorised into three groups. **Excluders** are unable to accumulate or slightly accumulate HM in their tissues regardless of

the concentration of HM in the soil/growing medium. **Accumulators -** accumulate HM in their tissues in proportion to the HM concentration in the soil, and **hyperaccumulators** are unique to individual HM, and can accumulate HM in their tissue at higher concentrations than the soil. Brooks and Wither, (1977) first introduced the term hyperaccumulator when they observed *Rinorea bengalensis* to accumulate 17,500 ppm (dry weight) of Ni in their above-ground biomass parts and defined plants accumulating > 1000 ppm HM in their above-ground biomass as hyperaccumulators. On the other hand, van der Ent et al., (2013) proposed the criteria for defining a hyperaccumulator varies for different metals (100 ppm for Se and Tl; 300 ppm for Cu, Co, and Cr; 3,000 ppm for Zn in dried plant biomass).

1.5 Phytoremediation of chromium

During the last two decades, there has been significant research to elucidate the adaptation, accumulation and uptake mechanisms of Cr(III) and Cr(VI) by plants (Wakeel and Xu, 2020; Sinha et al., 2018; Shahid et al., 2017). In recent years, an increasing amount of literature on the phytoremediation of Cr has been accomplished at the laboratory scale, usually using greenhouse-based, hydroponic cultures with a range of chromium concentrations (Srivastava et al., 2021; Das et al., 2020; Ranieri et al., 2020; Wakeel and Xu, 2020; Sinha et al., 2018) (Sinha et al., 2018). The use of hydroponics allows for a relatively simple system focusing on rhizofiltration; however, it is important to understand the chromium removal process (phytoextraction) from contaminated soil (Wakeel and Xu, 2020; Shahid et al., 2017). Untreated chromium effluent from industries pollutes the riverbank sediment and soil; thus, identifying the phytoremediator for contaminated soil is essential.

1.5.1 Hyper-accumulators

Zhang et al., (2007) introduced *Leersia hexandra* as a potential Cr hyper-accumulator. In their study, Cr(VI) reduction and sequestration have been observed in hydroponic (batch) culture, and the highest bioaccumulation coefficients have been found on the leaves: 486.8 after 45 days of treatment at 60 ppm Cr(III) and bioaccumulation coefficients 72.1 after 45 days treatment at 10 ppm Cr(VI). Chromium (total) accumulated in foliage was 4868 ppm for Cr(III) treatment and 597 ppm for Cr(VI) treatment (Zhang et al., 2007). However, while grown in ≥ 20 ppm of Cr(VI), a substantial decrease in foliage biomass have been observed (Zhang et al., 2007). pH is an important factor for Cr(VI) speciation and Cr(III) availability, but the author did not mention the pH of the hydroponic system. In another Cr(III) (as $CrCl_3$) phytoremediation study, Liu et al., (2011) applied fertiliser for the growth of *Leersia hexandra* and the author observed 45% growth in biomass and 26% more Cr extracted by *Leersia hexandra* compare to the results of plant growos without fetiliser. Finally, *Leersia hexandra* swartz has been considered a potential plant for phyto-extraction, especially in large-area of (low-concentration) contaminated environment (Lin et al., 2018; Zhang et al., 2007).

Redondo-Gómez et al., (2011) identified *Spartina argentinensis* (Cordgrass) as a hyperaccumulator of Cr(VI) while studied in a greenhouse (batch) condition grown in an inert substrate (pearlite) for six months and allocated to six Cr(VI) treatments (0 to 20 mmol/L). Results showed that plants accumulated 15100 ppm (DW) Cr(VI) in plant tissue after 15 days with 20 mmol/L of Cr(VI).

Gardea-Torresdey et al., (2004) introduced *Convolvulus arvensis* (Bindweed) herbaceous perennial plant of the Convolvulaceae family, as a promising hyperaccumulator for Cr(VI). In his experiment, the author germinated the seeds of

C. arvensis in spiked (0 and 80 ppm of Cr(VI)) agar-based nutrient mediums. The accumulated Cr in roots was about 20,000 ppm (DW) Cr(VI) and 2100 ppm (DW) Cr(VI) in foliage when allowed to grow in an agar-based nutrient medium spiked with 20 ppm Cr(VI) for two weeks. The author suggested *C. arvensis,* a hyperaccumulator for hexavalent chromium based on the accumulation at 20 ppm. ***Prosopis laevigata*** (smooth mesquite) also showed a high accumulation of Cr(VI) while seeds were germinated in tissue (batch) culture condition in modified Murashige–Skoog medium added with $K_2Cr_2O_7$ (0-353.6 ppm) at pH of 5.8 (Buendía-González et al., 2010). After 50 days, accumulation in roots reported 8090 ppm Cr(VI) (DW), and shoots contained 5461 ppm (DW) Cr(VI). However, the translocation factor of chromium was calculated below 0.7 but due to high accumulation in root author suggested ***Prosopis laevigata*** as a hyperaccumulator (Buendía-González et al., 2010).

1.5.2 Plant Selection for Phyto-extraction

The plants recognised as suitable for accumulation of HM, while it accumulates high concentrations (> 1000 ppm) of HM on its aerial (above soil biomass) part or low accumulation of HM with high biomass (growth rate) (Varun et al., 2017; USEPA, 2000; Ali et al., 2013). Besides this, many factors have been identified which control the phytoremediation potentiality of a plant. After horizon scanning with literature, the following characteristics need to be considered before selecting a plant species for phyto-extraction study.

- High yield and more arial part biomass.
- Well-distributed root system.
- Higher accumulation of HM (targeted) from contaminated environment.

- Capacity to grow in higher level of selected contaminants.
- Well adapted to local (where phytoremediation is needed) weather.
- Have higher resistance to plant pathogens and insects.
- Less effort on cultivation and harvesting after phytoremediation.
- Nonedible to humans and animal helps to prevent food adulteration.

1.6 Factors Affecting Phytoremediation of Chromium

There are various factors that affect the phytoremediation of chromium from contaminated soil. Some are endogenic (plant characteristics) and some are exogenic (media or environment) factors. The major exogenic factors are discussed below.

1.6.1 pH

It has been extensively reported that the speciation of chromium changes with pH (Figure 2). Cr(VI) is available in nature at a wide range of pH but especially as chromate in a basic medium (CrO_4^{2-}) and dichromate in an acidic medium ($Cr_2O_7^{2-}$) (Shewary and Peterson, 1976; Xu et al., 2020).

Previous research has shown changing pH from 3 to pH 6.7 showed higher (1.5 times) accumulation of Cr(VI) while barley seedlings in hydroponic culture (Shewary and Peterson, 1976). Cary et al. (1977) studied changes in pH from 5.0 to 8.0 while grown in Cr(VI) by wheat (Cary et al., 1977) and observed an increase in uptake as pH increased from 5.0 to 6.0, but they observed a decrease in Cr(VI) uptake from pH 6.0 to 8.0. These studies indicate that pH significantly impacts Cr(VI) uptake by plants.

1.6.2 Soil organic matter and root exudates

Oxidation states and mobility of chromium in soil are affected by soil organic matter (Alyazouri et al., 2020; Hayat et al., 2012; Choppala et al., 2018). The degraded organic matter and root exudates produce dissolved organic carbon (DOC) and low molecular weight organic acids (LMWOAs) (e.g. citric, oxalic acid, salicylic acids), which lowers the pH of soil. These DOC and LMWOAs convert Cr(VI) to the less bioavailable form Cr(III) (Chiu et al., 2009; Farid et al., 2017; Jean et al., 2008), but in contrast, the formation of the oxidised chelating ligands (LMWOAs from root exudates) form complexes with Cr(III), making them bioavailable (Hayat et al., 2012).

For example, Srivastava et al., (1999) studied *Lycopercicum esculentum* under Cr(III) stress condition and observed that accumulation of Cr(III) was enhanced in roots by carboxylic acid and amino acids (root exudates). In addition, researchers have found that due to its cationic nature, Cr(III) is chelated by LMWOAs (form root exudates) and then precipitated within the rhizosphere (Mishra and Tripathi, 2009; Suñe et al., 2007).

In summary, many studies have suggested that organic matter has the capacity to reduce Cr(VI) to Cr(III), which is less toxic to plants. By contrast, soil organic acids (LMWOAs) make Cr(VI) more bioavailable to plants. Therefore, measuring the amount of organic matter and DOM (dissolved organic matter) in contaminated soil before and during the phytoremediation is essential.

1.6.3 Effect of Chelating Agents

Chelating agents (e.g., ethylenediaminetetra-acetic acid and citric acid) can increase the solubility of HM in soils ((Evangelou et al., 2007)) and have also been

applied in phytoremediation studies to increase the accumulation of HM by plants (Ram et al., 2019; Mahmood-ul-Hassan et al., 2017; Farid et al., 2017).

In a study, *Ipomonea aquatic* (water spinach) was exposed to Cr(III) with or without EDTA (ethylenediaminetetra-acetic) at pH 6 under hydroponic conditions. The addition of EDTA improved the Cr(III) accumulation in roots by forming Cr-EDTA complexation, but these complexes hindered the translocation of Cr(III) from root to shoot (Chen et al., 2010).

Jean et al., (2008) investigated the phytoremediation efficiency of *Datura innoxia* for Cr(III) uptake from industrial-contaminated soil with the aid of EDTA and citric acid. The study results showed that citric acid was more effective than EDTA with a 2 and 3.5 x increase in translocation factor (TF) compared to the control (Jean et al., 2008).

The effect of natural low-molecular-weight organic acids (LMWOAs) and EDTA on Cr phytoextraction (soil containing 3100 ppm of Cr(III)) have been studied with *Brassica juncea,* and the author recommended LMWOAs over synthetic chelators (SC) because SC reduces plant shoot biomass and is not suitable for the environment (Hsiao et al., 2007).

Hybrid Napier grass (*Pennisetum americanus* L. × *Pennisetum purpureum* Schumach) was grown in soil with different concentrations of Cr(VI) (0, 20, 40, and 60 ppm) with and without EDTA (4mM) (Ram et al., 2019). The bio-accumulation factor (BAF) and translocation factor (TF) of Cr(VI) in plants increased with higher Cr(VI) concentration. EDTA increased the Cr(VI) accumulation in the root; however, due to total chromium being measured rather than Cr(VI), it was not possible to determine whether EDTA affected the speciation and uptake of Cr (Ram et al., 2019).

The effects of EDTA on the accumulation and translocation of two forms of chromium on *Salix matsudana (*hybrid willow) plants and *Salix babylonica* (weeping willow) plants were investigated by Yu and Gu, (2008) in a hydroponic system (at 24.0 ± 1°C) spiked with either potassium chromate (Cr(VI)) or chromium chloride (Cr(III)). Under control conditions with EDTA, uptake of Cr(III) was 3x higher than Cr(VI) in both willow species, but there was an insignificant translocation factor recorded for both Cr(III) and Cr(VI). However, researchers also reported that weeping willow with EDTA did not increase the uptake of Cr(VI) but showed elevated translocation of Cr(VI), although both plants were from the same genus (Yu and Gu, 2008).

In summary, it is difficult to come to a definitive conclusion regarding the role of chelating agents in the phytoremediation of Cr(VI), as the amount and types of chelating agents required for phytoremediation vary from species to species.

1.6.4 Effect of Sulfur

Sulfur (S) plays a vital role not only in animal nutrition but also in plant growth, and it also helps plants to assimilate N (e.g., as ferredoxins), which are essential for plant growth (Ihsan et al., 2019; Hawkesford, 2000; Resurreccion et al., 2001).

Plants uptake chromium via a sulphate transport mechanism by the addition of adequate sulfur also helps to manage Cr-induced plant stress (Kulczycki and Sacała, 2020; Alyazouri et al., 2020; Sardella et al., 2019; Holland and Avery, 2011). Alyazouri et al., (2020) irrigated *Portulaca oleracea* (purslane) with sulfate (300–600 ppm) and observed higher chromium uptake (compared to control) due to the activation of sulfate transporters in the root and while irrigated with sulphate (> 600 ppm), sulphate transporters in the root were saturated with sulphate anion, and a

reduction in the Cr(VI) uptake in the root was recorded (Alyazouri et al., 2020). Previous research has shown that Cr (as chromate) uptake by duckweeds (*Spirodela polyrhiza* and *Lemna minor*) was affected by sulphate, with Cr uptake increased at a low level of sulphates (1.25 ppm) but prevented at ≥ 960 ppm (high concentration) (Appenroth et al., 2008). These studies suggested that at high concentrations of sulphates, the bonding capacity of the sulphate transporters in roots was exceeded, with preferential binding of S over Cr (Appenroth et al., 2008). In another study, *Pteris vittata* improved its accumulation of Cr in the root by 1.3 – 7.8 times under moderate concentrations (~125–250 ppm) of sulphate in the growth medium, but no significant translocation was observed from root to fronds (de Oliveira et al., 2016; De Oliveira et al., 2014). The plant cell membrane contains the sulfate transport gene (e.g., SHST1) (Lindblom et al., 2006). Lindblom et al., (2006), genetically modified by the initiation of SHST1 gene in *Brassica juncea* (Indian mustard) (common hyper-accumulator) and observed higher accumulation in the root (46.5%) and in shoots (66%) compared to control in a hydroponic system containing 5ppm of Cr(VI) (as potassium chromate). However, compared to Cr(III) transportation and uptake, Cr(VI) transportation and uptake is metabolically driven with plant cellular sulphate transporters because of the resemblance in geometry, charge and size of both sulphate and chromate ion (Schiavon et al., 2008; Appenroth et al., 2008; Holland and Avery, 2011).

In early studies, researchers considered sulphate as a strong inhibitor of Cr(VI) uptake (Shewary and Peterson, 1976; Kleiman and Cogliatti, 1997). By contrast, in recent studies, the uptake of chromium has been shown to be enhanced by low concentrations of sulphates in growth media and reduced at high concentrations (Kulczycki and Sacała, 2020; Alyazouri et al., 2020; Sardella et al., 2019; Holland

and Avery, 2011). However, the application of sulfur in plants' phytoremediation study of Cr(VI) is still poorly known. Thus, understanding the sulphate-chromate interaction before phytoremediation for individual plant species is essential.

1.6.5 Effect of rhizobia legume symbiosis

Soil supports uncountable microorganisms, and among them, plant-growth-promoting bacteria (PGPB) accelerate plant growth and development and also help plants to cope with numerous biotic and abiotic (e.g., HM) stresses (Ahemad, 2015; Fagorzi et al., 2018). By contrast, PGPB enhances the Cr uptake capacity of plants and the phyto-availability of Cr in soil by generating several primary and secondary metabolites (e.g., siderophores and organic acids) (Braud et al., 2009; Fagorzi et al., 2018; Dimkpa et al., 2009). In addition, the bioavailability of Cr also increases in the presence of bio-surfactants released by bacteria in soil because it drains the strongly bonded metal from the soil (Gnanamani et al., 2010; Sheng et al., 2008). Din et al., (2020) studied the effects of *Bacillus xiamenensis* PM14 on *Sesbania sesban* grown in Cr-contaminated soil and observed double positive effects. The inoculant (*B. xiamenensis*) improved plant growth under Cr stress and increased Cr accumulation compared to the uninoculated plant. In addition, Jobby et al., (2019) observed Cr(VI) resistant *Sinorhizobium* sp. SAR1 bacteria could transform Cr(VI) in the bacterial cell wall to Cr(III). In summary, PGPB in legume plants produces numerous primary and secondary metabolites, which increase the bioavailability of Cr in soils and enhance the accumulation rate of Cr in the plant (Stambulska et al., 2018; Ahemad, 2015). However, the accumulation of chromium varies with species of plant and rhizobacterium.

1.7 *Sesbania cannabina* (Retz.) Poir.

Dhaincha (local name in Bangladesh) (Sesbania spp.) belongs to the family Leguminosae (sub-family Papilionoideae). *S. cannabina* is used as a green manure crop because of its high growth, is readily decomposed (requires less water), increases total carbon and nitrogen in the soil, and controls soil erosion (Sarwar et al., 2015; Ahmed et al., 2009).

Figure 1.6: Sesbania cannabina (Retz.) Poir. in laboratory condition.

It can be found in areas with a semi-arid to sub-humid climate, with rainfall between 500 and 2000 mm per year and a temperature of 18–23°C. Because of its tolerance to low temperatures, *S. cannabina* can grow at high altitudes (100 m to 2300 m), and it can also grow in waterlogged, flooded, saline, acidic, and alkaline soils. (Degefu et al., 2011; Sarwar et al., 2015; Chavan and Karadge, 1986).

As discussed in section 1.5.2, *S. cannabina* might be an ideal candidate for phytoremediation of chromium-contaminated soil.

1.7.1 Phytoremediation of HM by species of Sesbania genus.

The phytoremediation potential of several species of the *Sesbania* Genus for different HM has been studied. Chromium toxicity on *Sesbania sesban* (L.) Merr has been studied, and the inhibitory effect was witnessed with increasing levels of Cr compared to the control, but less effect was observed in germination percentages (Bakiyaraj et al., 2014). Patra et al., (2020) studied the phytoremediation potential between *Sesbania sesban L.* and *Brachiaria mutica* and found *S. sesban* has higher bioaccumulation and tolerance capacity while grown in chromium-contaminated soil (92.5 ppm Cr). In another study, the researcher found in *Sesbania sesban* that if the Cadmium concentrations in the soil exceeded 1.00 g /kg, cadmium caused the reduction of morpho-physiological (low biomass, low height) parameters in treated plants rather than control plant (Soundararajan and Veeraiyan, 2014). Varun et al., (2017) suggested *Sesbania sesban* (L.) as a potential candidate for phytoremediation of Cd from polluted soil, and in this experiment highest accumulation have been observed in roots (86.7 ± 6.3 ppm), stem (18.59 ± 1.9 ppm), and leaf (3.16 ± 1.1 ppm) while grown in 300ppm Cd dosed soil.

Sesbania exaltata was relatively tolerant of lead (McComb et al., 2012), and *Sesbania drummondii* was considered a hyperaccumulator of lead (Pb) under hydroponic culture in greenhouse conditions (Barlow et al., 2000). In another study, *Sesbania cannabina* showed a high accumulation of different HM while allowed to grow on various amounts of fly ash-containing soil, and the highest accumulation of Iron (Fe) was observed in plant tissue (Sinha and Gupta, 2005).

1.8 Summary and Gap of the chromium phytoremediation studies:

The plant toxicity of Cr(III) is very low compared to Cr(VI) (Sinha et al., 2018; Pradas del Real et al., 2020); however, some phytoremediation studies were conducted only on Cr(III) (Raimondi et al., 2020; Barbosa et al., 2007; Coelho et al., 2017; Mant et al., 2005) and authors incorrectly made a conclusion that these plants were able to remove chromium (Cr(III) and Cr(VI)) from contaminated land. It is crucial to understand the effect of Cr speciation (e,g. Cr(III) or Cr(VI)) on phytoremediation potential, but in some studies, a mix of Cr(III) and Cr(VI) contaminated soil or wastewater (mostly from tannery effluents) were used (Khilji and Firdaus-e-Bareen, 2008; Vymazal et al., 2007; Patra et al., 2020). Again, these studies incorrectly reported Cr accumulation in plants as Cr(VI) or total chromium because they did not analyse Cr speciation in plant tissue, soil or wastewater. In other studies, researchers have applied an unrealistically low concentration of Cr(VI) (≤ 10 ppm) (Karimi, 2013; Kale et al., 2015; Vymazal et al., 2007; Choo et al., 2006).

Most of the phytoremediation research has been designed using hydroponic systems because hydroponic systems (rhizofiltration) are more controllable experiments than others (Augustynowicz et al., 2010; Zhang et al., 2007; Uysal, 2013). However, results must be applied to the environment with caution; plants that demonstrate accumulation of Cr(VI) in hydroponic systems need to also be tested in contaminated soil. Some studies use a nutrient medium (agar-based), Murashige–skoog medium or inert substrate (pearlite) instead of soil or hydroponic systems (plant grown in water-based nutrient solution) in their research and present the plant as a potential hyperaccumulator, ignoring the effects of heterogenous growing medium (Buendía-González et al., 2010; Gardea-Torresdey et al., 2005; Redondo-Gómez et al., 2011).

It is well-established that pH and soil organic matter substantially affect chromium speciation (figure 1.5), but many Cr phytoremediation studies fail to mention the effects of pH and soil organic matter (Zhang et al., 2007; Patra et al., 2020). Sulfur (as sulfate) acts as a competitive nutrient, and the gene (SHST1) responsible of the uptake of S also competes with Cr. This competition is plant species and concentration-dependent and needs further investigation (Chotchutima et al., 2016; Alyazouri et al., 2020; Kulczycki and Sacała, 2020). In recent studies, chelating agents (e.g. EDTA) have been applied to increase the removal efficiency of chromium by plants, but this mechanism is restricted to the chelation of Cr(III) by EDTA (Adiloğlu and Göker, 2020; Jean et al., 2008; Revathi and Subhashree, 2019).

1.9 Aim and Objectives

This study aims to assess the phytoremediation potential of *Sesbania cannabina* for removing Cr(VI) from the soil with contamination typical for Bangladesh.

To achieve this aim the following objectives are made:

1. To identify and assess the extent of Cr contamination in riverbank sediments of the Buriganga and Dhaleshwari river of Bangladesh.
2. To determine the effect of Cr(VI) on seed germination of *S. cannabina.*
3. To observe the root growth while subjected to different concentrations of Cr(VI) in soil.
4. To determine the effect of Cr(VI) concentration on the uptake of chromium by *S. cannabina* in soil and determine its potential as an accumulator or species capable of phyto-sequestration.

1.10 Outline of the Thesis

This thesis is divided into 7 chapters, a brief summary of each chapter is provided below :

Chapter 2

Subtitle : An assessment of distribution and concentrations of total chromium, chromium (VI) and lead (Pb) in river sediments during the dry season after the relocation of the tannery industries from the Buriganga to the Dhaleshwari Rivers in Bangladesh.

This section of the study aims to assess Cr (total), Cr(VI) and lead (Pb) concentration in the riverbank sediment of the Dhaleshwari with a comparison of the Buriganga river because the government are shifting the tannery industries from the bank of the Buriganga to the bank of the Dhaleshwari river. We collected sediment samples in the winter of 2019 and 2021.

Chapter 3

Subtitle: Optimisation of seed germination and seedling emergence of *Sesbania cannabina* (Retz.) Poir.

This study aims to optimise the condition for seed germination and seedling emergence of Sesbania cannabina for a phytoremediation study. In this experiment, seed germination was carried out using two growth media: Murashige and Skoog basal medium (MS); and Whatman Grad 1 filter paper (FP), using the top of media or top of the paper method under three different photoperiods.

Chapter 4

Subtitle: Effect of hexavalent chromium on seed germination and root development of *Sesbania cannabina* (Retz.) Poir. in two different growth media.

This study aims to determine whether S. cannabina can be grown from seed under hexavalent chromium-stressed conditions to establish this species as a tool for phytoremediation. In this experiment, toxicity testing of hexavalent chromium ($K_2Cr_2O_7$) on seed germination was carried out using two growth media: Murashige and Skoog basal medium (MS); and Whatman Grade 1 filter paper (FP) spiked with different concentration of Cr(VI), by the top of media or top of paper method respectively.

Chapter 5

Subtitle: Evaluation of the root system of *Sesbania cannabina* grown in hexavalent chromium contaminated soils by utilising modified rhizobox systems.

This study aimed to assess how the root system of Sesbania cannabina behaves under various concentrations of Cr(VI) and whether it could be a suitable species for the phytoremediation of Cr(VI) contaminated soils. The experiment was conducted in rhizoboxes under greenhouse conditions using a sandy loam soil dosed with potassium dichromate giving eight different Cr(VI) concentrations (0 ppm, 5 ppm, 10 ppm, 20 ppm, 40 ppm, 80 ppm, 160 ppm, and 360 ppm). Plant roots were photographed with a Canon 60D (18-megapixel) camera with a 50 mm prime lens and analysed with Image J image processing software.

Chapter 6

Subtitle: An assessment of the phytoremediation potential of *Sesbania cannabina* grown in hexavalent chromium contaminated soil.

In this study section, we have discussed about the Cr(VI) phytoremediation capacity *S. cannabina*. To assess the growth, tolerance, and phytoremediation ability of *S.*

cannabina, a pot experiment was conducted under greenhouse conditions (simulated tropical conditions), and *S. cannabina was* grown in Cr(VI) spiked soil.

Chapter 7

Conclusion

This section summarises the main findings and recommends further study on *Sesbania cannabina* for phytoremediation and other applications.

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Chapter 2 :

An assessment of distribution and concentrations of total chromium, chromium (VI) and lead in river sediments during the dry season after the relocation of the tannery industries from the Buriganga to the Dhaleshwari Rivers in Bangladesh.

Abstract

Chromium (Cr (total) and Cr(VI), and lead (Pb) are the most toxic heavy metal (HM) known to humans. These HM are usually released into the environment from industries and transport systems. In Bangladesh, most of the urban river becomes polluted due to industrial activities. This study aims to assess Cr (total), Cr(VI) and lead (Pb) concentration in the riverbank sediment of the Dhaleshwari with a comparison of the Buriganga river because the government are shifting the tannery industries from the bank of the Buriganga to the bank of the Dhaleshwari river. We collected sediment samples in the winter of 2019 and 2021. The presence of Cr(VI) in the riverbank sediment of the Dhaleshwari river indicates active pollution from tanneries; however, Cr(VI) was observed at ≤ 1 ppm in 2021 in the riverbank sediment of the Buriganga river, which was previously known to be the most toxic site for chromium. Lead (Pb) concentration did not vary between the year (2019 and 2021).

Keywords: Cr(total), Cr(VI); lead(Pb); Dhaleshwari river; Buriganga river; riverbank sediment.

2.1 Introduction

Bangladesh is a great delta formed by the alluvial deposits of the three mighty Himalayan Rivers: the Ganges (as the Padma in Bangladesh), the Brahmaputra (as the Jamuna in Bangladesh) and the Meghna; these river systems drain a total catchment area of about 1.72 million sq km through Bangladesh into the Bay of Bengal (Whitehead et al., 2015; Rasul, 2015; Kolås et al., 2013a). There are 170 million people that live beside the 405 rivers of Bangladesh, among which 57 are trans-boundary rivers. The life and livelihood of agriculture-based Bangladeshi people have been revolving around waters of these rivers over the ages (Whitehead et al., 2015; Rasul, 2015; Kolås et al., 2013a). After the industrial revolution in late 1990s in Bangladesh, wastewater from industries discharged into many rivers causing rapid, significant degradation of water and sediment quality. The extent of water pollution varies with season and during winter or dry seasons these rivers become more polluted than during the monsoon (Islam et al., 2015; Bhuiyan et al., 2015; Islam et al., 2014; Kolås et al., 2013b).

Dhaka is Bangladesh's capital, surrounded by three rivers, the Buriganga, Turag and Shitalakhya. Economic growth and rapid population growth in Dhaka, has caused an increase in various wastes (including wastewater from industries) polluting the surrounding rivers of the capital. Among these rivers, the Buriganga is one of the most polluted rivers in Bangladesh. Major polluting industries such as tanneries and textile industries are situated on the bank of the Buriganga River and discharge untreated or partially treated wastewater into the river (Islam et al., 2018a; Nargis et al., 2018; Islam et al., 2015). Tannery industries, located in Hazaribagh, Dhaka, are one of the major pollution sources of the Buriganga River (Asaduzzaman et al., 2016; Tamim et al., 2016). In the era of the fourth industrial revolution, the

Government of Bangladesh (GoB) planned to relocate these industries to a well-structured tannery industrial park at the bank of the Dhaleshwari River (a tributary of the Buriganga) in Hemayetpur, Savar on the outskirts of Dhaka (NEWAGE, 2022; Islam et al., 2021; Roy and Akash, 2018). The important aims of this relocation was to reduce the pollution magnitude of the Buriganga River and to release the treated tannery wastewater from the central effluent treatment plant of newly developed tannery cluster into the Dhaleshwari River to avoid detrimental effects for the aquatic ecosystem because in the Hazaribag area tanneries were built unplanned way (NEWAGE, 2022; Islam et al., 2021; Roy and Akash, 2018) . The government of the People's Republic of Bangladesh started the relocation process in 2003, but it has yet to move the last remaining tanneries from Hazaribagh and so far (upto December 2019) 139 tanneries out of 154 (in Hazaribag) are now in operation in the industrial park (NEWAGE, 2019).

Chromium sulphate ($Cr_2(SO_4)_3 \cdot x(H_2O)$, where x can range from 0 to 18) is used in the main process of tanning in leather processing industries, and around 10 kg of chromium sulphate has been used in the tanning processes for 100 kg of salted wet hide (Ludvík, 2000). Leather processing industries at Hazaribag and Kamrangir Char of Dhaka city (figure 2.1) were the main reason for the heavy metal (chromium) pollution in the Buriganga River as the industries discharge their untreated or partially treated effluents directly into the river (Islam et al., 2018b; Nargis et al., 2018; Bhuiyan et al., 2015; Saha and Hossain, 2011). Since Cr salt is an essential chemical for the tanning process, we can assume that the presence of excessive Cr in the environment might originate from the tannery industries.

Many studies have assessed the extent of river water pollution surrounding the capital Dhaka (mostly the rivers Buriganga, Turag and Shytalakhya), but very few

have investigated sediment quality (Nargis et al., 2018; Islam et al., 2018b, 2016; Sikder and Islam, 2016). More recently, researchers have been interested in assessing and monitoring the pollution status of the Dhaleshwari River with a concern that it has similar degraded conditions as the Buriganga River. However, most of the work in the Dhaleshwari River has focused on water quality (Islam et al., 2021; Hasan et al., 2020). In addition, all the past studies on both water and sediment quality only estimated total Cr and did not report the status of Cr(VI) or Cr(III) for the tannery-polluted rivers in Bangladesh. Beside Cr, it was reported that the wastewater from tannery industry also might contain other metals such as zinc, iron, cadmium, arsenic, lead, nickel and copper (Das et al., 2011). Sediment contamination is of importance as pollutants can be stored and then remobilised over time, particularly during periods of high flow, causing secondary pollutant effects (Yi et al., 2011; Islam et al., 2018b). The present study provides a determination of Cr(VI), total Cr and lead (Pb) contents in the riverbank sediment of the Dhaleshwari and the Buriganga Rivers. Here, beside Cr we selected Pb as a surrogate of other heavy metals as it was one of the most polluting metals in the rivers of Bangladesh. This allowed us to check whether the relocation of one type of industry would have an influence on Cr and Pb distribution in the two studied rivers. Therefore, the main objective of the study was, to assess the chromium (total and Cr (V)) and lead (Pb) loads in river sediments with temporal and spatial variations.

2.2 Methodology

2.2.1 Sediment sample collection

Sediment samples were collected from eight sites (figure 2.1) of the Dhaleshwari (L1 to L4) and the Buriganga Rivers (L5 to L8) (these sampling sites in Buriganga river have been selected after a literature review to compare with other research (varies around 50 meters from previous studies discussed in table 2.5) during the dry season of December 2019 and December 2021. Samples were collected within 1 m from the water line of the rivers using a hand augur. The top ~5 cm of collected sediment in the augur was rejected, and the next ~10 cm of sediment was collected. The samples were put in a pre-washed polyethene bag which was put in another polyethene bag with a label. The samples were kept in the icebox in the field and later preserved in the freezer at below – 4° C, and we transferred the samples from Bangladesh to the laboratory of the University of Birmingham (UK); the frozen samples were stored in the - 15°C until metal determination.

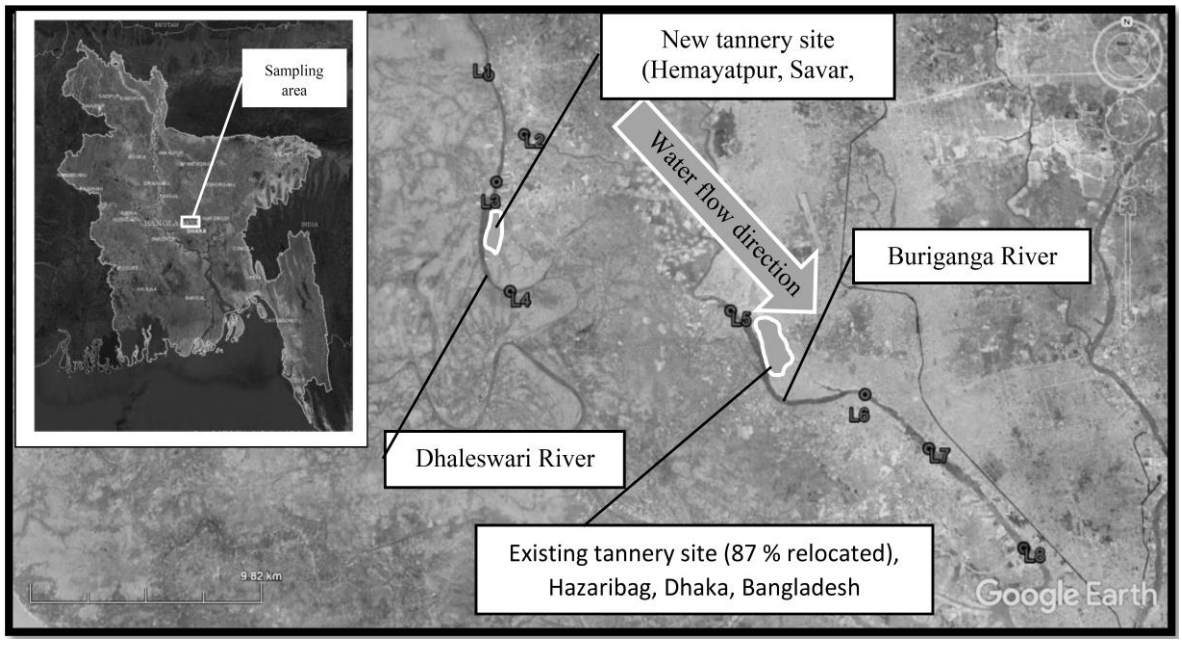

Figure 2.1: Sample collection location points in both rivers.

2.2.2 Sample preparation and Cr analysis

Sediment samples were dried at 65 °C for at least 48 hours to a constant weight and then ground using a mortar and pestle (Alyazouri et al., 2020).

2.2.2.1 Analysis of total chromium (Cr) and lead (Pb)

Samples were digested with acid [(Acid digestion: oven-dried samples were crushed, and 0.1 g of powdered (homogenised) samples were placed into a digestion tube with 23 ml of HCL (con.) and 7 ml of HNO_3 (con.). After 12 hours, the digestion tube was placed in a heating block at 80° C for reflux for 2 hours and then filtered with a wetted filter (Whatman 41) paper into a 25 ml volumetric flask. An ionisation suppressant KCl (0.5 ml of 10%) is added to the filtered materials, and finally, the tube and filter paper are repeatedly washed with ultra-pure water and made volume up to the mark of a volumetric flask)]; and the resulting solution analysed with ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy; model: Agilent 7500ce, detection limit ≥ 0.001 ppm). All chemicals were ICP grade.

2.2.2.2 Analysis of hexavalent chromium (Cr(VI)

Hexavalent chromium: samples were digested [(alkaline digestion: 2.5 ± 0.10 g of the sample were placed into a clean 250 mL digestion vessel and 50 mL ± 1 mL (dissolved 20.0 ± 0.05 g NaOH and 30.0 ± 0.05 g Na_2CO_3 in reagent water in a one-litre volumetric flask and diluted with DI water and maintained the pH 11.5 or greater,)] of digestion solution were added to each digestion vessel. We also added approximately 400 mg of MgCl and 0.5 mL of 1.0 M phosphate buffer mixed for 5 min. After that, the digestion vessel containing the samples was heated to 90-95° C

(for 60 min). After cooling, the contents (in a beaker) were transferred quantitatively to the filtration apparatus and filtered through a 0.45µm membrane filter. Slowly add 5.0 M nitric acid solution to the beaker dropwise and adjust the solution's pH to 7.5 ± 0.5. Transferred the vessel's contents quantitatively to a 100 mL volumetric flask and adjusted the sample volume to 100 mL (to the mark for the volumetric flask) with reagent water and mixed well. The sample digestates are now ready to be analysed, and the resulting solutions were analysed using a JENWAY spectrophotometer 6505 following EPA method 3060A and method 7196 (USEPA, 1996; De Oliveira et al., 2014; Alyazouri et al., 2014).

2.2.3 Estimation of geo-accumulation index

Müller (1979) introduced the geo-accumulation index (*I*geo) to assess the anthropogenic impact of contaminants. It is estimated using the following equation:

$$I\text{geo} = \log_2 \frac{C_n}{1.5B_n}$$

where C*n* is the measured metal concentration in the sediment, B*n* is the background concentration of the metal and 1.5 is the factor compensating background data (correction factor) due to the lithogenic effect (Taylor 1964). Here, average shale concentration was used as the background concentration of the metal given by Turekian and Wedepohl (1961).

The geo-accumulation index is composed of seven grades. Class 0 (uncontaminated): *I*geo ≤ 0; Class 1 (uncontaminated to moderately contaminated): 0 < *I*geo < 1; Class 2 (moderately contaminated sediment): 1 < *I*geo < 2; Class 3 (moderately to strongly contaminated): 2 < *I*geo < 3; Class 4 (strongly contaminated): 3 < *I*geo < 4; Class 5 (strongly to extremely contaminated): 4 < *I*geo < 5; Class 6 (extremely contaminated): 5 ≤ *I*geo.

2.2.4 Estimation of contamination factor

The contamination factor (CF) is used as global standard reference for unpolluted sediment. It was calculated by comparing the mean of estimated metal concentration with average metal concentration in shale as given by Turekian and Wedepohl (1961). It was calculated by the following equation:

$$CF = \frac{\text{Mean metal concentration at contaminated site}}{\text{Metal average shale concentration}}$$

Hakanson (1980) classified CF values into four grades, i.e., $CF < 1$ in Class 1 with low CF, $1 \leq CF < 3$ in Class 2 with moderate CF, $3 \leq CF < 6$ under Class 3 with considerable CF and $CF \geq 6$ kept in Class 4 with very high CF.

2.2.5 Statistical analyses

Generalised linear mixed models (GLMMs) were used to determine the difference in metal concentrations between the Buriganga River and the Dhaleshwari River. Here, the response variable was the metal concentration, the explanatory variable was river, the random effect was the sampling years, error distribution was Gaussian with an identity link function.

Likelihood-ratio test was performed to establish the influence of sampling years on metal concentrations according to the sampling site. A full model was developed with the response variable of metal concentration and the explanatory variables sampling year and sampling sites and their interaction. The reduced model was developed without the interaction term between the explanatory variables. Error distribution for all models was Gaussian with the identity link function. Full and reduced models were compared in likelihood-ratio test. If the result was significant, it suggested that sampling year have an influence on the sampling locations means

tannery relocation has an impact on the concentration of chromium in the riverbank sediment. Multiple comparisons were done for each year to find out which sampling sites were specifically influenced. Here, in the developed GLMMs the response variable was metal concentration, the explanatory variable was sampling sites of the rivers and the error distribution was Gaussian with an identity link function.

The significance level (α) was set at $p = 0.05$. In multiple comparisons, α was adjusted using Bonferroni correction dividing by the number of sampling years ($p = 0.025$). All statistical analyses were performed using the "glmmADMB" and "multcomp" packages in R (Version 3.6.2; R Core Team, 2019).

2.3 Results

The Buriganga River had significantly higher Cr (total) and Pb concentrations in the sediments than the Dhaleshwari River (tables 2.1 and 2.2, figure 2.2). Year had a significant effect on metal concentrations in sediments of both rivers (table 2.3). For Cr (total) concentrations in the Dhaleshwari River L2 and L4 sites and in the Buriganga River L6 and L7 sites had significantly different concentrations between 2019 and 2021 whereas for Pb there was no significant difference between 2019 and 2021 for all sites in both rivers (figure 2.3).

In the Dhaleshwari River Cr (total) in L4 site had the highest concentrations for both sampling years and in the Buriganga River L6 and L7 had the highest concentrations in 2019 and 2021, respectively (table 2.1 and figure 2.3). For Pb, in the Dhaleshwari River L1 site had the highest concentrations for both sampling years and in the Buriganga River L7 site had the highest concentrations for both sampling years (table 2.1 and figure 2.3).

Chromium (VI) concentrations were below the detection level in most of the sampling sites of both rivers for both sampling years (table 2.1), hence no statistical analysis was performed for Cr(VI). In the Dhaleshwari River only L4 site had detectable Cr(VI) concentrations in 2019 and 2021, and in the Buriganga River only L6 site showed detectable Cr(VI) concentration in2019. This trend was similar with Cr (total) in L4 and L6 sites in 2019 and 2021. Therefore, it might be possible to assume that Cr(VI) concentrations would be high where Cr (total) concentrations were high and vice-versa.

Table 2.1. Chromium (total), Cr(VI) and Pb concentrations (ppm) in the sampling sites in the Dhaleshwari River (L1 to L4) and in the Buriganga River (L5 to L8) in 2019 and 2021 (mean ± SD).

Location	**Dhaleshwari River**				**Buriganga River**			
	L1	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**
2019								
Cr (total)	69.67±1.69	94.67±1.25	90.33±13.02	125.33±3.09	116.33±3.39	331±6.16	304.33±4.92	200.33±7.13
Cr(VI)	< 1	< 1	< 1	24.66±4.18	< 1	47.66±4.49	< 1	< 1
Pb	34	27	19.67	17	14.33	27.33	221.67	201.67
2021								
Cr (total)	85.66±5.44	114.33+3.3	129+2.16	302.33±13.6	109.33±4.99	129±2.45	233±8.64	212.33±4.03
Cr(VI)	< 1	< 1	< 1	31.67±2.87	< 1	< 1	< 1	< 1
Pb	35±2.16	19±1.63	21.67±2.05	14.67±1.7	12.68±1.7	20±2.16	266.34±12.26	192±5.89

Table 2.2. Summary statistics of generalised linear mixed models (GLMMs) to determine difference in metal concentrations (ppm) in sediments between the Buriganga and Dhaleshwari Rivers (n indicates sample size).

Response variable	n	Explanatory variable	Coefficient	Standard Error (SE)	*p*-value
Cr (total), ppm	48	River	-78	21.4	<0.001
Pb, ppm	48	River	-96	21.1	<0.001

The models showed the Buriganga River had higher metal concentrations (also see, figure 2.1).

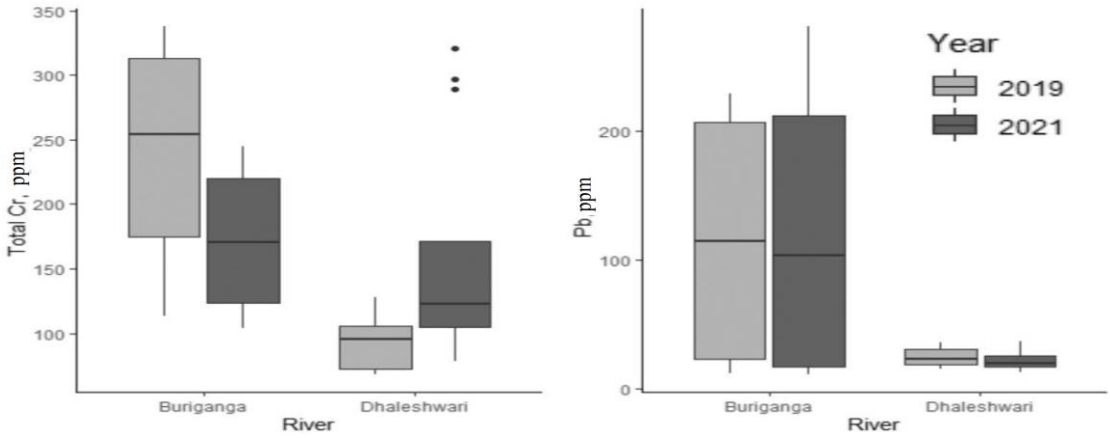

Figure 2.2. Total Cr and Pb concentrations (ppm) in the Buriganga and Dhaleshwari Rivers in 2019 and 2021. Boxplot legend: top (bottom) edges of box are 75th (25th) percentiles; center line in the box is median; the upper (lower) whisker extends from the box edge to the largest (smallest) value no further than 1.5 × inter-quartile ranges of the edge; data beyond the end of the whiskers are outliers and are plotted individually)

Table 2.3. Summary statistics of the likelihood-ratio test to examine the effects of interaction between sampling year (Y) and sampling site (S) on the Cr (total) and Pb concentrations (ppm) in the Dhaleshwari and the Buriganga Rivers. (*df* indicates the degrees of freedom).

Response variable	Model type	Explanatory variables	*df*	Log-likelihood	Deviance	*p*- value
Dhaleshwari River						
Cr (total)	Full	Sampling year (Y) × Sampling site (S)	3	-81.4	74.59	<0.001
	Reduced	Sampling year (Y), Sampling site (S)		-118.69		
Pb	Full	Sampling year (Y) × Sampling site (S)	3	-49.37	17.39	<0.001
	Reduced	Sampling year (Y), Sampling site (S)		-58.07		
Buriganga River						
Cr (total)	Full	Sampling year (Y) × Sampling site (S)	3	-75.19	97.44	<0.001
	Reduced	Sampling year (Y), Sampling site (S)		-123.91		
Pb	Full	Sampling year (Y) × Sampling site (S)	3	-76.46	36.66	<0.001
	Reduced	Sampling year (Y), Sampling site (S)		-94.79		

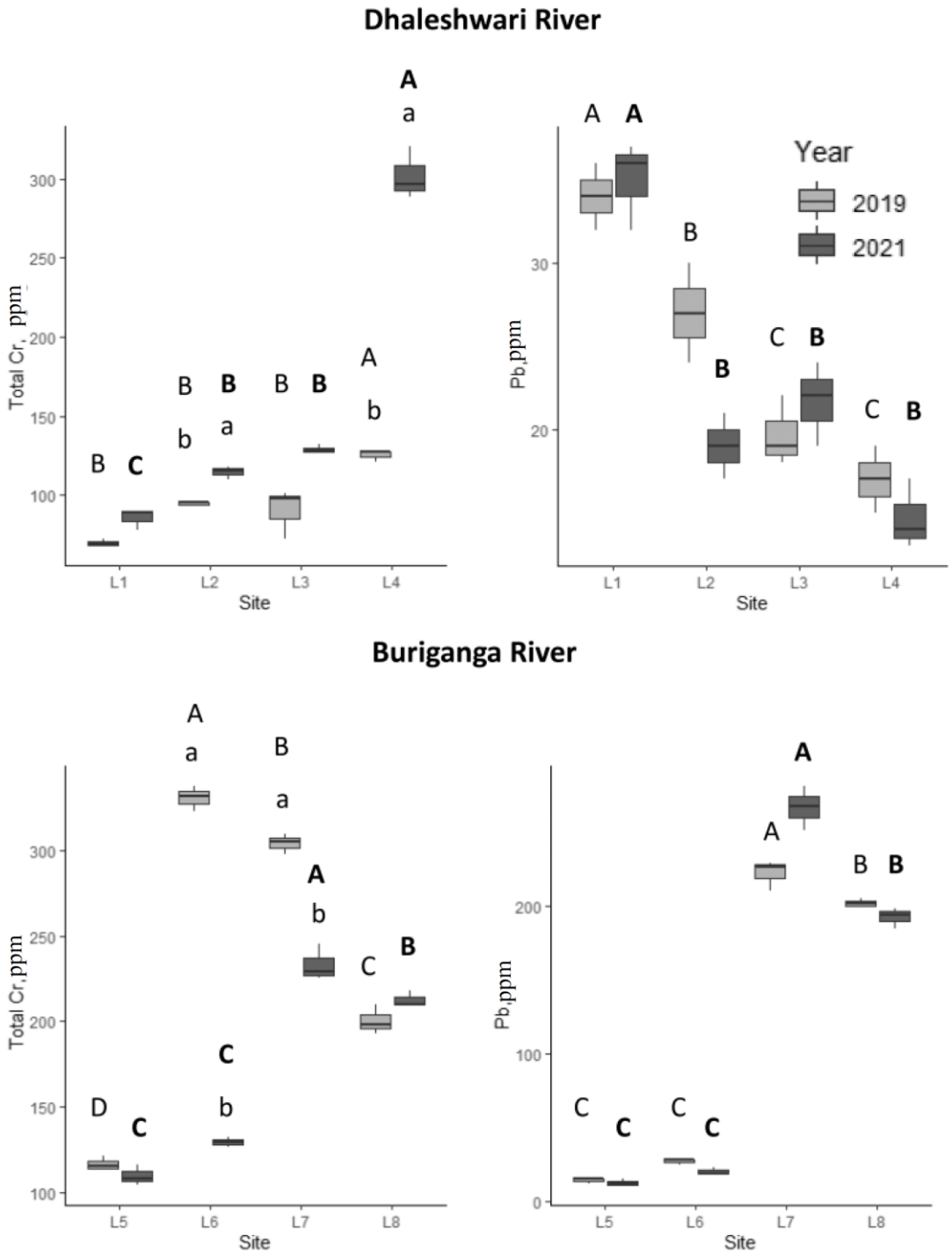

Figure 2.3. The Cr (total) and Pb concentrations (ppm) in the sediments of the sampling sites in the Dhaleshwari and Buriganga Rivers for 2019 and 2021. Small letters indicate the difference in metal concentrations between years (corrected p = 0.0125); and capital letters and bold capital letters indicate the difference between sites in each river for 2019 and 2021, respectively (corrected p = 0.025)

Table 2.4. Summary of geo-accumulation index (Igeo) and contamination factor (CF) for Pb and total Cr in the Dhaleshwari and Buriganga rivers in 2019 and 2021 (see figure 2.1 for sample locations L1 to L8). L1 to L4 upstream and L5 to L8 downstream).

Year	Site	River	Geo-accumulation index				Contamination factor			
			Pb		Total Cr		Pb		Total Cr	
			mean	SD	mean	SD	mean	SD	mean	SD
2019	L1	Dhaleshwari	0.18†	0.08	-0.95*	0.04	1.70†	0.10	0.77*	0.02
2019	L2	Dhaleshwari	-0.16*	0.16	-0.51*	0.02	1.35†	0.15	1.05†	0.02
2019	L3	Dhaleshwari	-0.61*	0.15	-0.60*	0.27	0.98*	0.10	1.00†	0.18
2019	L4	Dhaleshwari	-0.83*	0.17	-0.11*	0.04	0.85*	0.10	1.39†	0.04
2019	L5	Buriganga	-1.08*	0.22	-0.22*	0.05	0.72*	0.10	1.29†	0.05
2019	L6	Buriganga	-0.14*	0.11	1.29‡	0.03	1.37†	0.10	3.68‡	0.08
2019	L7	Buriganga	2.88‡‡	0.07	1.17‡	0.03	11.08‡‡	0.51	3.38‡	0.07
2019	L8	Buriganga	2.75‡‡	0.02	0.57†	0.06	10.08‡‡	0.15	2.23†	0.10
2021	L1	Dhaleshwari	0.22†	0.11	-0.66*	0.11	1.75†	0.13	0.95*	0.07
2021	L2	Dhaleshwari	-0.66*	0.15	-0.24*	0.05	0.95*	0.10	1.27†	0.04
2021	L3	Dhaleshwari	-0.48*	0.17	-0.07*	0.03	1.08†	0.13	1.43†	0.03
2021	L4	Dhaleshwari	-1.04*	0.20	1.16‡	0.08	0.73*	0.10	3.36‡	0.19
2021	L5	Buriganga	-1.26*	0.23	-0.31*	0.08	0.63*	0.10	1.21†	0.07
2021	L6	Buriganga	-0.59*	0.19	-0.07*	0.03	1.00†	0.13	1.43†	0.03
2021	L7	Buriganga	3.15‡‡‡	0.08	0.79†	0.06	13.32‡‡	0.75	2.59†	0.12
2021	L8	Buriganga	2.68‡‡	0.05	0.65†	0.03	9.60‡‡	0.36	2.36†	0.05

Here, SD is the standard deviation; number of samplings in each site was three. For Igeo, * indicates uncontaminated sites with class 0, † indicates uncontaminated to moderately contaminated with class 1, ‡ indicates moderately to strongly contaminated with class 3, ‡‡ indicates strongly contaminated with class 4 and ‡‡‡ indicates strongly to extremely contaminated with class 5. For CF, * indicates class 1 with low CF, † indicates class 2 with moderate CF, ‡ indicates class 3 with considerable CF and ‡‡ indicates class 4 with high CF.

Geo-accumulation index and contamination factor (table 2.4) of total Cr in L6 and L7 sites showed the highest values for the Buriganga River in 2019, which decreased notably in 2021. In the site furthest downstream (L8), the differences of all indices between the two years were minimal for total Cr. In the Dhaleshwari River, all indices values increased between 2019 and 2020 from upstream to downstream sites (i.e., L1 to L4). However, in the L4 site, a notable increase occurred from 2019 to 2021 for all indices.

Geo-accumulation index and contamination factor of Pb showed the highest values in L7 site, followed by L8 site for the Buriganga River in 2019 and 2021. In 2021, the indices values of Pb in L7 site increased from 2019, whereas in the L8 site, it decreased. In the Dhaleshwari River, the most upstream site (L1) had the highest indices values for Pb in 2019 and 2021.

2.4 Discussion

The Buriganga River has been heavily polluted with total Cr and Pb for more than a decade and previous research has reported these two elements to be of major concern (Islam et al., 2018b; Nargis et al., 2018; Bhuiyan et al., 2015; Saha and Hossain, 2011). In 2009, Mohiuddin et al., (2011) observed 243 ppm Cr in monsoon and 109.0 ppm Cr in winter in sediment samples from the Buriganga River. These values were about 4 times higher than the study of 2015-2016 as reported by Nargis et al. (2018) (table 2.5). According to Bhuiyan et al., (2015) the concentrations were very high in the winter and the variation was between 1715 to 1019 ppm. In another study in 2013, the highest chromium concentration was 841 ppm (in L5 site) and the lowest value 22 ppm observed in downstream (in L7 site) during winter (Islam et al., 2018b).

Table 2.5: Total chromium (ppm) contents in the sediments of Buriganga River (Sampling site varies around 50 meters for each researcher).

Location		Sampling time	2021	2019	2016	2015	2014 (Jan)	2013	2012	2010	2009
Bosila Bridge	**L5**	Feb –Mar			70.81			841	2471	1535±38	
		Aug–Sept				76.44		826	2039		
		December	109.33±4.99	116.33±3.39							
Badamtoli Ghat	**L6**	Feb- Mar			45.07		108.4	78	750	1715±31	149
		Aug-Sept				42.01		51	650		154
		December	129±2.45	331±6.16							
Near Pagla	**L7**	Feb- Mar			39.4		187.3	22		1019±25	243
		Aug-Sept				35.42		17			188
		December	233±8.64	304.33±4.92							
Fatullah	**L8**	Feb- Mar			33.57					1020±76	
		Aug-Sept				32.58					
		December	212.33±4.03	200.33±7.13							
		Source:	This study	This study	(Nargis et al., 2018)	(Nargis et al., 2018)	(Mohiuddin et al., 2015)	(Islam et al., 2018b)	(Islam et al., 2014)	(Bhuiyan et al., 2015)	(Mohiuddin et al., 2011)
		Methods	ICP-OES	ICP-OES	ICP-MS	ICP-MS	AAS	ICP-MS	ICP-MS	EDXRF	ICP-MS

In the most recent study during 2015-2016 the mean concentrations of Cr varied from 39.70 ± 18.84 (monsoon) and 41.45 ± 15.88 ppm (winter) (Nargis et al., 2018). In comparison our present study showed mean concentrations of total Cr of 237.99 ± 85.54 and 179.91 ± 52.72 ppm in the winter of 2019 and 2021, respectively. There is no doubt that total Cr is still present in the riverbank sediment of the Buriganga river. However, the distribution pattern of total Cr concentrations showed a common trend in all studies, i.e., at downstream of the tannery industries, total Cr concentrations continuously decreased. For example, Nargis et al., (2018), Bhuiyan et al., (2015) and the current study found that total Cr concentrations continuously

decreased from L6 (the closest downstream site of the tannery industries; see figure 2.1) to L8 sites (table 2.5).

Total Cr concentration in the Buriganga River might decrease after shifting the tannery industries, as reported by several past workers (Correspondent, 2017; Roy, 2017) and the present study also supported this possibility. Total Cr concentration in L6 and L7 sites (i.e., the two consecutive closest downstream sites of the tannery industries; see figure 2.1) decreased from 2019 to 2021. Although past workers did not report Cr(VI) concentration in the sediment of the Buriganga River to our best knowledge, among all Cr species Cr(VI) has the most detrimental effects for living organisms (ADD REF). In 2019, we only observed mean (±SD) Cr(VI) concentration as 47.67 (±4.49) ppm in the closest downstream site of the tannery industries (i.e., L6 site; see figure 2.1), whereas in 2021 Cr(VI) concentration was <1 ppm. This may also suggest that moving the tanneries resulted in Cr(VI) pollution because more active tanneries were present in 2019 than in 2021 in the bank of Buriganga river. However, in the Dhalashwari river, we observed an elevated level of Cr(VI) in the riverbank sediment at point L4 (table 2.1).

The present findings are compared with previous studies in the same location (± 50 m) of the Buriganga river (table 2.5). In all locations in Buriganga river, total chromium varies between 331±6.1 ppm to 109.33±4.9. According to Canadian Sediment Quality Guidelines for the Protection of Aquatic Life, in all locations, total chromium is higher than the permissible limit; according to them, interim sediment quality guidelines (ISQGs) is 37.3 ppm and probable effect levels (PELs) for chromium 90 ppm (CCME, 1999a). Bangladesh has no standard for riverbank sediment or freshwater sediment quality guideline for chromium like many other countries.

Table 2.5 shows the summary results of the chromium concentration in the same sampling point (±50 m distance) between 2019 and 2021. Chromium concentration in the sediment of the Buriganga river decreases over time but in some places, the changes were negligible (L5 and L8) (Islam et al., 2018b; Nargis et al., 2018; Bhuiyan et al., 2015; Saha and Hossain, 2011).

In the Dhaleshwari River, the highest total Cr concentration was found in L4, the closest downstream site of the relocated tannery industries. Total Cr concentrations in the riverbank sediment of the Dhaleshwari River increased over time between 2019 and 2021, for example, at L4 Cr concentration was 125.33±3.09 ppm in 2019 compared to 302.33±13.6 ppm in 2021 (as shown as outliers in figure 2.2). It therefore seems possible that increased total Cr concentration are linked to the relocated tannery industries on the bank of the Dhaleshwari River. However, sites upstream of the tannery industries (L1, L2 and L3) also had elevated total Cr concentrations in 2021 compared to 2019. There was no geological changes occurred in the upstream of the sampling locations so the increasement might not be for geogenic reason. The possible anthropogenic reason might be increase in Cr-releasing chemical usages in agriculture and textile industries. The upstream of the Dhaleshwari River has agricultural land coverage and the river receives treated, untreated or partially treated wastewater from several textile industries. After the revocation of lock-down due to the Covid-19 pandemic situation, the acceleration in agricultural and textile industrial activities might be the cause of such enhanced total Cr concentrations in the upstream sites of the tannery industries.

Past workers observed lead (Pb) in the riverbank sediment of the Buriganga river above the permissible limit in most cases (Mohiuddin et al., 2011; Nargis et al., 2018). According to Canadian Sediment Quality Guidelines for the Protection of

Aquatic Life (1999) in location L7 (221.67 ppm in 2019 and 266.34±12.26 ppm in 2021) and L8 (201.67 ppm in 2019 and 192±5.89 in 2021) lead concentration in sediment was higher than the permissible limit, according to them interim sediment quality guidelines (ISQGs) is 35 ppm and guideline also stats that probable effect levels (PELs) for lead is ≥ 91.3 ppm (CCME, 1999b). In our study, Pb concentration was observed to be higher than these levels in location L7 (between Badamtali ghat and Pagla) and L8.

The Buriganga River had higher Pb concentrations than the Dhaleshwari River; however, Pb did not follow the same distribution among the sites like total Cr and Cr(VI), which is likely due to Pb being a minor contaminant from tannery industrial processes. Several past studies (Mohiuddin et al., 2011; Nargis et al., 2018) reported the presence of high Pb concentration in the Buriganga River due to the presence of surrounding unplanned battery, steel and lead pipe recycling factories that discharged Pb-rich wastewater in the river. Additionally, the Buriganga River is a major shipping route for transporting goods and passengers (Alam, 2008), and there are several small ship-making and repairing shipyards from where Pb can be released to the river from leaded paints, gasoline etc. During the field visit in the Dhaleshwari River, the authors observed a big municipal unsanitary waste disposal site containing e-waste and several textile, pharmaceutical, and ceramic tiles industries adjacent to the upstream sites river. Along with these point sources, it was possible to have other point sources, which might be the sources of Pb that caused high Pb concentration in the Dhaleshwari River. For example, lead-acid battery-driven three-wheeler vehicles are very common mode of transportation in the semi-urban areas in Bangladesh (Nargis et al., 2018; Alam, 2008) which was also observed in the surroundings of the sampling locations as well as the upstream

localities of the two rivers. The unauthorised lead-acid battery recycling factories in those areas might be another possible source of Pb.

In this area (near L7) Mohiuddin et al., (2011) observed the highest concentration of Pb (474.85 in monsoon and 477.85 ppm in winter), Ahmad et al., (2010) reported only 67.45 ppm in the monsoon and 69.02 ppm in the winter and Nargis et al., (2018) recorded 45.22 ppm during the winter.

The Buriganga River is more polluted than the Dhaleshwari River. We found an unpleasant odour coming from the Buriganga River water during sampling and the chemical analyses also supported a higher pollution load than the Dhaleshwari River. One positive finding for the Buriganga River in this study would be the decrease of metal loads (i.e., total Cr and Cr(VI)) from 2019 to 2021, which were compulsorily used in the tannery industrial processes. On the contrary, total Cr loading in the sediment of the Dhaleshwari River increased from 2019 to 2021. In case of Cr(VI), we could not perform statistical analysis due to lower detection level for the most samples; however, we found a slight increase of mean (±SD) Cr(VI) concentration only in L4 site from 2019 (24.66±4.18 ppm) to 2021 (31.67±2.87 ppm). Although the relocated tannery industries have a common effluent treatment plant, the presence of Cr in the sediment suggests that these are not sufficient to remove the metal contaminants. As more and more tannery industries are relocated in the tannery industrial park, it is likely that Cr loading in the Dhaleshwari River sediment will increase without additional treatment measures. The shift in potential pollution could be understood more appropriately from the geo-accumulation index. In the Buriganga River the index was better graded for total Cr, whereas in the Dhaleshwari River it was worse graded from 2019 to 2021 at the closest downstream sites of the tannery industries (i.e., L6 and L4 sites, respectively).

This study did not determine the metal concentrations in the river water. The metal concentrations in water usually vary significantly in a short period due to seasonal effects, changes in release from the pollution sources, variations of chemical reaction rate factors amongst others (Nargis et al., 2018; Islam et al., 2018a, 2018b). The metal concentrations in sediment are generally more stable, which might provide a better indication of the long-term anthropogenic metal loads in the environment. For example, the higher concentration of chromium in sediment than in water is generally found due to the properties of insolubility, lower mobility and higher bonding capacity with organic matter in soil and sediment with the species of Cr^{3+} oxides, hydroxides, and sulphates (Becquer et al., 2003; Peralta-Videa et al., 2009).

There has been previous research on metal concentrations in the Buriganga River (table 2.5). However, it was difficult to directly compare the metal concentrations among various studies. Despite having a common pattern in metal distribution (see earlier discussion), the metal concentration varies from one study to another. The possible reasons might be due to (a) variation in the season which was clearly present in the data records of the workers (Islam et al., 2014; Islam et al., 2018b; Mohiuddin et al., 2011; Nargis et al., 2018) who analysed samples for two seasons; (b) variation in sampling locations, e.g., the distance the water line could influence the metal concentration in the sediment and (c) variation in analytical methods, e.g., various workers used different analytical instruments such as ICP-OES, ICP-MS, AAS and EDXRF (table 2.5).

2.5 Conclusion

From this study, it has been confirmed that the Dhaleshwari river bank sediment is now being contaminated with chromium due to tannery effluent despite the central effluent treatment plant in tannery facilities. However, chromium pollution in the Buriganga river is declining (based on riverbank sediment data). On the other hand, lead (Pb) pollution was only observed in the riverbank sediment of the Buriganga river.

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Chapter 3

Optimisation of seed germination and seedling emergence of *Sesbania cannabina* (Retz.) Poir.

Abstract

Assessment of seed germination and seedling emergence are essential steps for toxicity testing and phytoremediation studies. The life cycle of a plant depends on seed germination and seedling emergence. *Sesbania cannabina,* a leguminous fodder crop with rapid growth and high biomass production, is naturally growing in many areas worldwide and may have phytoremediation potential. This study aims to optimise the condition for seed germination and seedling emergence of *Sesbania cannabina* for a phytoremediation study.

In this experiment, seed germination was carried out using two growth media: Murashige and Skoog basal medium (MS); and Whatman Grad 1 filter paper (FP), using the top of media or top of the paper method under three different photoperiods. Seeds were pre-treated for different lengths of time with various concentrations of hydrogen peroxide (H_2O_2) (v/v) multiple hot water treatments. Only imbibed seeds were considered for the germination study in all pre-treatment conditions. In addition, seed emergence was determined within soil and compost. Results showed that seeds pre-treated with H_2O_2 (6% v/v) for 5 minutes and primed with 65°C water for 5 minutes were considered as the ideal pre-treatment condition. Different photoperiods and media used in germination in this experiment do not significantly affect seed germination. The optimum condition for seed germination at 27.5 °C ± 2.5°C and a relative humidity of ~ 75% for 5 days. Seed emergence in soil and compost was significantly affected by the burial depth and bulk density of the media, with the highest (98%±1) seed emergence observed at 1 cm depth for soil and compost, and decreasing with increased burial depth. This ideal condition will help in further studies related to plant growth and phytoremediation of *S. cannabina.*

Key Words: Sesbania cannabina, germination, seedling emergence, hydrogen peroxide.

3.1 Introduction

The accumulation of heavy metals (HM) in the biosphere due to anthropogenic activities has become a widespread problem (Zwolak et al., 2019; Awa and Hadibarata, 2020; Vardhan et al., 2019). To date, there has been substantial research in the field of soil pollution, its effect on plants and phytoremediation, including the effects on seed germination and early stages of plant growth (Kuriakose and Prasad, 2008; Munzuroglu and Geckil, 2002). Phenotypic and morphological changes occur in response to various biotic and abiotic stressors (e.g. heavy metals) during seed germination, which is very important for the plant life cycle (Wojtyla et al., 2016). The actual effect of contaminants on seed germination is difficult to assess until 100 %, or maximum seed germination, is achieved before toxicological studies. Some research has failed to state the maximum germination condition in their research, providing an important limitation to the interpretation of these studies (i.e Zhi et al., 2015; Guterresa et al., 2019; Sahoo et al., 2018).

Numerous environmental factors affect the germination of wetland species, including daytime temperature variations, water availability, oxygen, flooding and shallow sediment cover (Lorenzen et al., 2000; Webb et al., 2009). For some types of seeds, seed cover hinders water penetration and prevents the embryo from growing (Shreelalitha et al., 2015; Chanda et al., 2017). Many plant species have been studied for phytoremediation of heavy metals and it is crucial to understand the seed germination condition before phytoremediation studies. It has been reported that *Sesbania* (genus) seeds of Fabaceae or Leguminosae family have such a seed coat that prevents imbibition and thus hinders germination (Guppy, 1912). There are several ways to increase seed germination rate in the presence of

a seed coat and among the most important are a) temperature (e.g. hot water treatment) (e.g.Iqbal et al., 2019) b) pre-treatment with Polyethylene Glycol (PEG 6000) (e.g.Muscolo et al., 2014) c) use of beneficial fungi (e.g. *Trichoderma harzianum*) (Bharath et al., 2005) d) seed disinfection (e.g NaClO or H_2O_2) (Iqbal et al., 2019; Chigbo and Batty, 2013) and e) use of Gibralic Acid (GA3) (Kołodziejek et al., 2017; Lawes and Anderson, 1980). Several techniques have been applied to increase the germination rate of legume species seeds, such as hot water treatment and physical or acid scarification (Dan and Brix, 2007).

Sesbania cannabina, an annual shrub, is commonly found in China, the Indian sub-continent, southeast Asia, Papua New Guinea, Australia and the South Pacific Islands (Sarwar et al., 2015). Summer environments are suitable for rapid growth (up to 3.5 m) and development; however, the plant can grow in spring and autumn (Rao and Gill, 1995; Sarwar et al., 2015). Each mature plant can produce around 1,200 pods, which contain about 24 thousand more or less dark green to brown, rod-like seeds (Rao and Gill, 1995; Sarwar et al., 2015). The present study aimed to determine suitable conditions for maximum germination of the seeds of *Sesbania cannabina.* To achieve maximum germination and seedling emergence, we have considered the following experiment steps (1) identification of the appropriate dose of disinfectants (H_2O_2) for seed before germination, (2) ambient temperature for hot water treatment of seed, (3) suitable media for germination, (4) ambient air temperature for seed germination, (5) photoperiod for seed germination, and (6) seedling emergence from different burial depth of seeds.

3.2 Methodology

It was essential to achieve maximum (99%±1%) germination under optimal conditions before studying the impact of abiotic stress on seed germination. During the germination study, radicle size ≥ 2 mm represents the successful germination of the seed (Adhikari et al., 2022; Vidak et al., 2022). Seed germination percentages were assessed on day five after incubation based on a combined germination count of replicates (n=6).

3.2.1 Seed Collection and Storage

For use in all experiments, seeds of *S. cannabina* were collected in 2018 from Shobuz Biz Bhar, Bangladesh, a locally reputed seed-selling company and stored under dry conditions at 4°C temperature (Webb et al., 2009) for six months before use (figures 3.1 A).

3.1 (A)

3.1 (B)

Figure 3.1: Images of *S. cannabina* seed 1(A) healthy seeds before treatment, 1(B) after imbibition of seeds treated with hot water (65° C)

3.2.2 Seed selection and preparation for germination

Seed selection was performed by examination under a dissecting microscope (Stereo Microscope SZ61TR), and deteriorated seeds (e.g., dirty or void seeds)

were rejected. Initially, selected seeds were then surface sterilised and treated with hot water. After treatment, seeds that appeared to be fully imbibed (water uptake by the dry seed) (figure 1. B) were selected for the germination experiment.

3.2.3 Effect of disinfectant and hot water treatment on seed germination

To determine the effectiveness of disinfectant on seed germination, healthy seeds were surface sterilised (soaked) in an orbital shaker (200 RPM, 5 minutes) with different concentrations (1%, 2%, 3%, 4%, 5%, 6%, 7% and 8%) of hydrogen peroxide (v/v) (Chigbo and Batty, 2013; Wojtyla et al., 2016). Following treatment, seeds were rinsed with de-ionised water and transferred to a petri dish (9 cm diameter). Five replicates of each treatment were prepared with 10 seeds per dish. These were incubated for five days at an air temperature of 25 ° C. After five days, a spot check for microbial growth was performed under the dissecting microscope. If any microbial growth was evident, this was considered positive (+) and where there was no visible microbial growth, negative (-). Minimum concentrations that showed maximum germination with negative results were considered a suitable disinfectant dose.

After surface-sterilisation (at a concentration discussed in 3.3.1), seeds were primed with different temperatures of hot de-ionised water temperatures of 50°C, 55°C, 60 °C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C and 100°C for 5 min. After treatment, seeds were placed on double-layer Whatman® 1 filter paper (FP). For each hot water treatment, 10 seeds were placed in each petri dish (9 cm diameter) on a growth medium (e.g., filter paper and MS media) with 6 replicates of each treatment.

3.2.4 Effect of growth media on seed germination

A wide variety of growth media have been used within germination experiments, including Murashige and Skoog basal medium (MS) (Siddiqui et al., 2014) and filter paper (moistened with 5 ml deionized water) (Chigbo and Batty, 2013) and Bacto agar (Bae et al., 2016). In this experiment, modified Murashige and Skoog basal medium (MS) and filter paper (FP) (Whatman Grad 1 filter) were compared for seed germination. Whatman Grade 1 filter paper (FP) meets the ISTA (International Seed Testing Association) requirement (Healthcare, n.d.). MS medium is also used in phytoremediation studies, especially for germination and seedling growth (Santiago-Cruz et al., 2014; Lusa et al., 2019). Both media did not contain any persistent, bio-cumulative or toxic compounds for plant growth (Buendía-González et al., 2010).

To determine how growth medium affects seed germination, 10 seeds were placed petri dish (in 9-cm-diameter, six replicates) on the growth medium (double layer of Whatman No. 1 filter paper (FP) and modified Murashige and Skoog basal medium (MS)). The growth media were then soaked with 5.0 ml of de-ionised water (Milli-Q® Gradient A10^{TM}), the petri dishes were wrapped with para-film to avoid evaporation and placed in a vitopod® propagator (temperature controlled, fixed at 28°C±1°C). In this experiment, MS was modified by adding sucrose and agar, modified MS medium containing 4.4 g MS, 30 g sucrose and 8 g nutrient agar per litre of medium (Buendía-González et al., 2010).

3.2.5 Effect of Temperature and Photoperiod

To understand the effect of temperature and photoperiod on seed germination, seeds were incubated at seven different fixed temperatures (5°C, 10°C, 15°C, 20°C,

25°C, 30°C and 35°C) under three different light treatments, **1**. darkness (24 h), **2**. light (24 h) and **3.** photoperiod (12/12-h). Petri dishes were covered with carbon paper to create darkened conditions for germination. All seeds were pre-treated with H_2O_2 and hot water (65°C).

3.2.6 Effect of burial depth for different growth medium on seedling emergence

Borosilicate glass cylinders (42mm diameter x 310mm height) with no drainage holes were filled to approximately 280 mm depth with growth medium (soil (~360 g) or compost (~120 g) and covered with dark paper. Two growth media were used in this experiment. The first was a sandy loam soil (supplied by Singletons Nurseries, UK) with a pH of 7.3, containing 2.1% organic matter 62, 87, and 412 kg ha^{-1} of N (nitrogen), P (phosphorus), and K (potassium) respectively. The second was compost (supplied by Singletons Nurseries, UK) with a pH of 5.3-5.8, containing ≥ 57 ± 2 % organic matter and 204, 104, and 339 kg ha^{-1} N,P,), and K , respectively. Before use, the soil/compost was autoclaved (to avoid contamination) and sieved with a 2-mm mesh net. Two seeds were placed at different depths for each treatment (with three replicates) and then covered with soil/compost to form depths of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 cm and irrigated with deionized water. A depth level of 0 cm (top of soil/compost) was excluded due to the risk of inadequate seed-soil/compost interaction and lower water uptake (Messersmith et al., 2000) and predation by pests (Chauhan et al., 2012). We consider seedling emergence while the shoots appear at the soil surface and were recorded every 24h from sowing up to 7 days.

3.2.7 Germination percentage

Germination percentage (TG_{hour})(at 12-hour intervals) was calculated using the formula (Bae et al., 2016):

$$TG_{hour} = \frac{\text{Number of germinated seeds at fixed hour}}{\text{Total number of seeds}} \times 100$$

3.2.8 Statistical analysis

Pearson Correlation (2-tailed) (correlation is significant at the 0.01 level) and One-way Analysis of Variance was carried out with SPSS (v 25). We checked normality and homogeneity of variance assumptions before ANOVA. When a significant ($p < 0.05$) difference was observed between treatments, multiple comparisons were made using the Tukey post-hoc test.

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of disinfectant and hot water treatment on seed germination

The results showed that ≥ 6% H_2O_2 (v/v) concentration was most suitable for the disinfection of *S. cannabina* seeds (table 3.1). A higher concentration (≥ 30 % v/v) of H_2O_2 has the capacity to damage tissues (Public Health England, 2009), and for that reason, 6% H_2O_2 is considered the most suitable concentration for seed disinfectants.

Hydrogen peroxide was previously recognised as a harmful chemical can damage cell or cell viability. Many studies have focused on the function of hydrogen peroxide in seed germination (Wojtyla et al., 2016; Barba-Espín et al., 2012); but the actual function of this molecule remains unknown. The main function of H_2O_2 in seed

germination is recognised as disinfection of the seed and as a signalling molecule for germination (Barba-Espín et al., 2012).

Table 3.1: Effect of different concentrations of H_2O_2 (where (+) means microbial growth and (–) is no microbial growth).

Dose (v/v)	Petri dish 1	Petri dish 2	Petri dish 3	Petri dish 4	Petri dish 5
1 %	+	+	+	+	+
2 %	+	+	+	+	+
3 %	+	+	+	+	+
4 %	+	+	-	+	-
5 %	-	-	+	-	-
6 %	-	-	-	-	-
7 %	-	-	-	-	-
8 %	-	-	-	-	-

In this section, after H_2O_2 (6%, v/v, 5 min) treatment, seeds were primed with different water temperatures and allowed to germinate in the germination chamber. Within 60 hours, the maximum germination was achieved under conditions of 65°C (98.2±1%) (pre-treatment for 5 min) (figure 3.2).

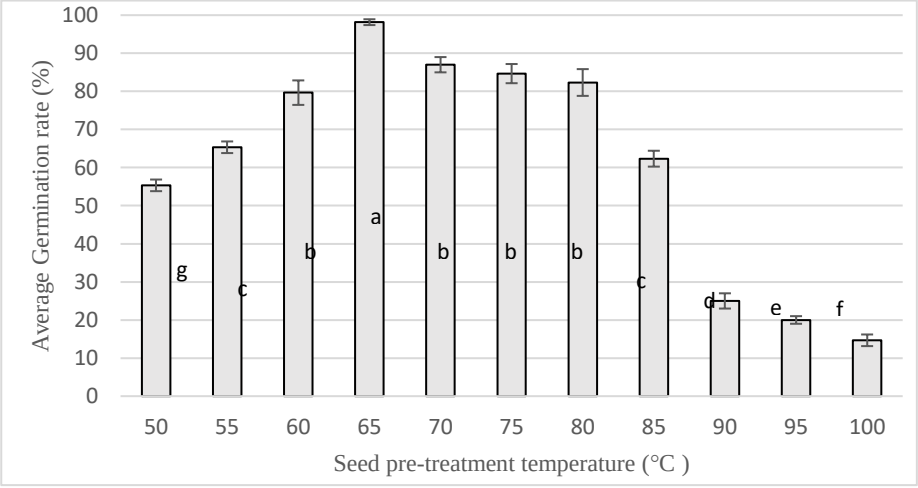

Figure 3.2: Effect of seed pre-treatment with hot water (for 5 min) on germination of *Sesbania cannabina* seeds at day 5. Error bars are standard error (n =5). Identical letters indicate no significant difference, as determined by Tukey's LSD ($p \leq 0.001$).

Due to low water permeability, the hard seed coat of *S. cannabina* makes the seed physically dormant, and this morphological characteristic makes these seeds resilient to various stressors (Veasey et al., 2000). The low temperature of the water is inadequate to soften the seed coat, while high temperature has a lethal impact on seed germination (Tarrega et al. 1992). Dan and Brix (2007) showed that 70ºC hot water pre-treatment produced a higher *S. sesban* seed germination rate than pre-treatment with 60ºC water or 98% H_2SO_4. In another study, seeds of *S. sesban* were first soaked in water at 80°C for 8 min to achieve (92±1.1%) germination (Wang and Hanson, 2008).

Iqbal et al., (2019) achieved 95 % ± 1 seed germination for *S. cannabina* by soaking seeds with sodium hypochlorite (NaClO) (1% v/v) for 1 min, followed by pre-treatment with boiling water (100±2 °C). In our experiment seeds treated with H_2O_2 (6%, v/v) showed higher germination percentages (99±1) than sodium hypochlorite (78±1) (Iqbal et al., 2019). In addition to that, the concentration used in our experiment has been found in many domestic (chlorine-free) bleach products and is more environmentally friendly than sodium hypochlorite (Public Health England, 2009, 2015; SCHER, 2008). 65°C hot water (for 5 min) was found to be the best suitable pre-treatment temperature for seed germination (figure 3.2). In this experiment, we observed maximum germination (in 5 days) with a combination of pre-treated with H_2O_2 (6%, v/v) and 65°C hot water (for 5 min).

3.3.2 Effect of growth medium on seed germination

No significant difference ($p > 0.05$) was observed on seed germination rate between two different media (figure 3.3).

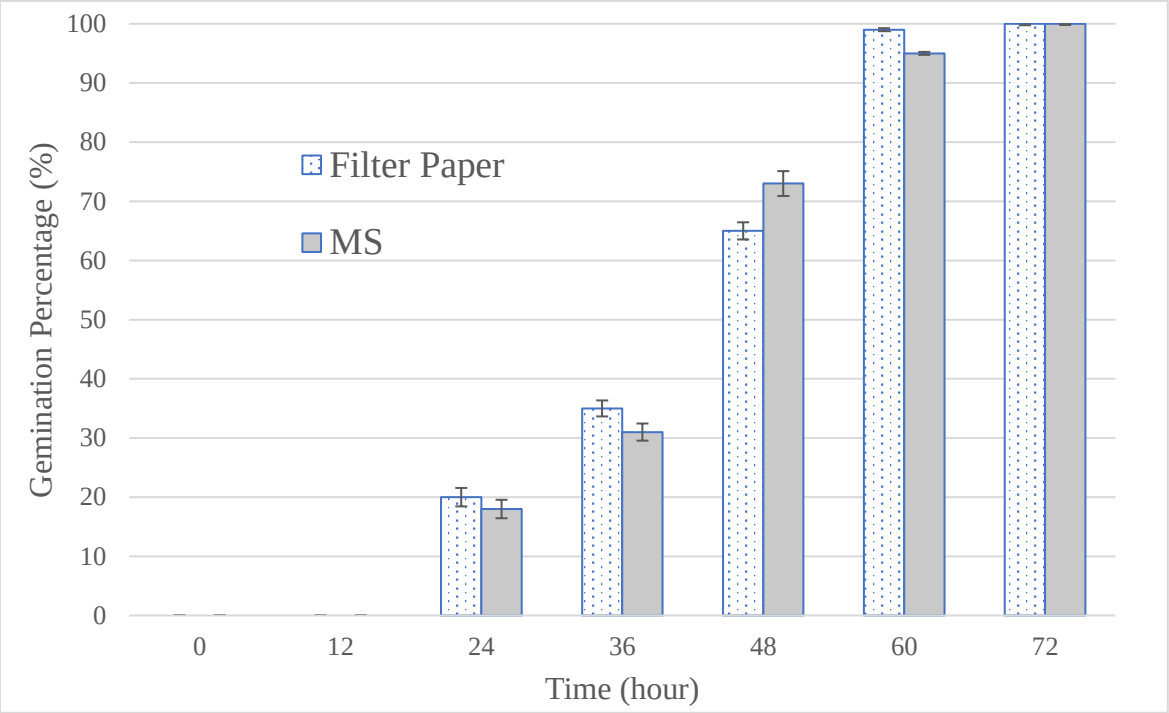

Figure 3.3: Seed germination on two different growth media. Error bars are standard deviation from the mean (n=5).

3.3.3 Effect of Temperature and Photoperiod

Seed germination at different temperatures (constant incubation temperatures) showed that temperature had a significant effect on germination ($p \leq 0.005$), and we also observed no significant effect of photoperiod on germination ($p > 0.05$) in each constant incubation temperature. In addition, only 0-4 % germination percentages were observed in 12/12-h photoperiod compared to day and night photoperiod thus, we can conclude that the seeds demonstrated a neutral photoblastic response (figure 3.4) where seeds can germinate in with or without light. Research has previously shown that dark and light conditions do not affect germination for seeds of *S. sesban* (Dan and Brix, 2007; Graaff and Staden, 1984). This may allow germination from greater burial depths (see figure 3.5).

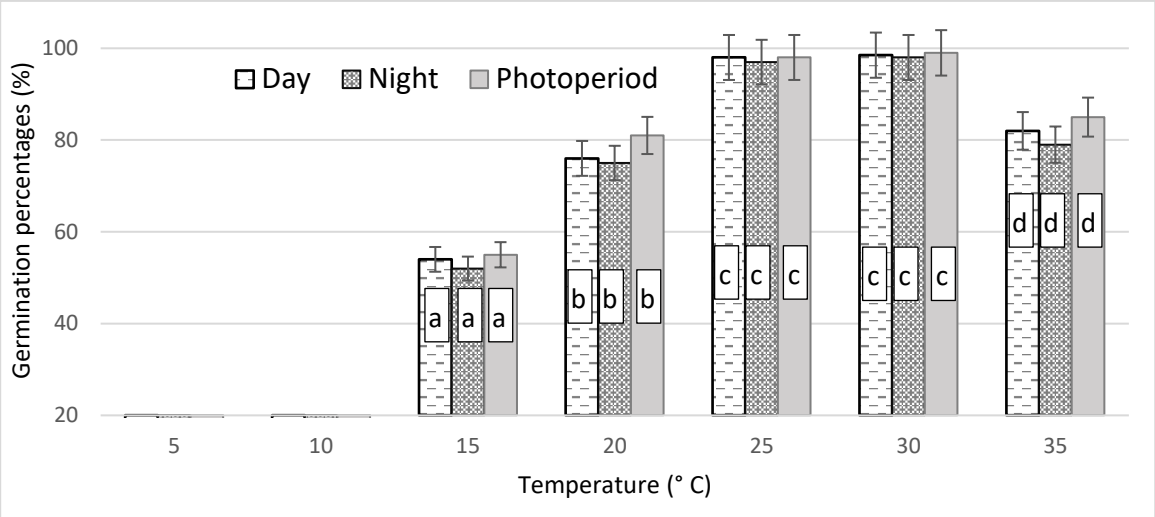

Figure 3.4: Effect of different (constant) temperatures and different photo-period on seed germination of for 5 d. Error bars are standard error (n =5). Identical letters indicate no significant difference, as determined by Tukey's LSD ($p \leq 0.001$).

The highest germination was recorded at temperatures between 25° C and 30° C, and germination percentages declined slowly as temperatures increased (> 30° C) or decreased (< 25° C) (figure 3.4). However, we observed no germination below 10° C. Enzyme activity and hormone synthesis during seed germination was impacted by the growth chamber or room temperature (Baskin and Baskin 2014). Thus, temperature (germination chamber or air) has a very high impact on germination. Research on *S. sesban* showed that the highest germination was observed at temperatures between 30 and 37 ºC, but germination stopped below 13 ºC or above 45 ºC (Dan and Brix, 2007). In a similar study, Iqbal et al., (2019) observed the highest germination of *S. cannabina* seeds (87%) at 32° C. *S. cannabina* seeds exhibit germination capacity in a wide range of temperatures, indicating that they have high adaptability and can germinate throughout the year in tropical countries. Hence, *S. cannabina* becomes more adaptive in diverse climates (15°C to 37°C).

3.3.4 Effect of burial depth within different growth media on seedling emergence

We observed a significant impact of varying burial depths on the seedling emergence of *S. cannabina* ($p < 0.001$) (figure 3.5), and the result showed that with an increase in burial depth, the seedling emergence rate declined. Comparing the two-growth media, the seed grown in compost exhibited more potential towards germination through different burial depths and recorded 9% higher germination than the seed germinated in soil. For both compost and soil, we observed maximum emergence (98±1%) for seeds buried under 1 cm depth, but with an increase in burial depth, seed germination and seedling emergence varied between the two media.

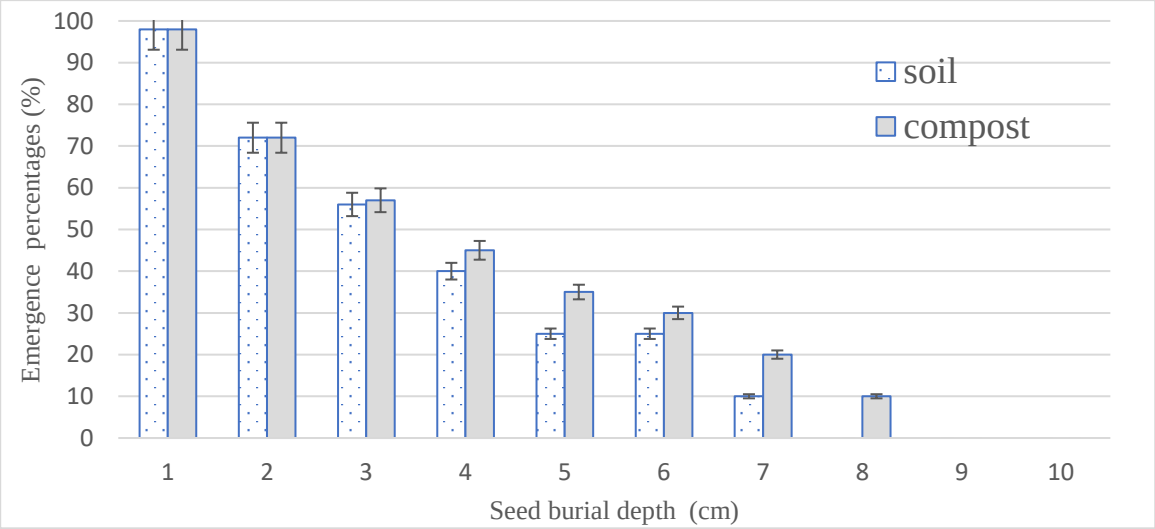

Figure 3.5: Seed emergence results of *S. cannabina* grown on soil and compost in a glasshouse at different depths, 28 C for 7 d. Error bars are standard deviation from the mean (n=5).

In this experiment, seeds buried ≥ 10 cm could not emerge within 7 days. We also observed that *S. cannabina* had the capacity to emerge from soil (≤ 7 cm) and compost (≤ 8 cm) within 7 days. Similar studies related to seedling emergence also show that seedling emergence had a negative correlation with seed burial depth

(Mennan and Ngouajio, 2006; Önen et al., 2018; Zhao et al., 2018). Stored food (carbohydrate reserve) within the seeds allows seeds to grow in dark conditions (Mennan and Ngouajio, 2006), but *S. cannabina* seeds have a moderate reserve compared to other plant species (Chanda et al., 2017), thus allowing the plant to grow from the deep burial (≤ 9cm).

3.4 Conclusion

Identifying viable seeds for assessing the phototoxicity of contaminants for germination studies is crucial. In summary, the results of this study demonstrate that *S. cannabina* can germinate and emerge within diverse environmental conditions. Treatment with 6% (v/v) H_2O_2 (5min) and 65°C hot water (5min), allowed seeds of *S. cannabina* to germinate rapidly under growth conditions of 25°C to 30°C. *S. cannabina* seed can emerge from burial depth in the soil (up to 8 cm) and compost (upto 9 cm) indicates that these seeds did not require any unique technique for cultivation.

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Chapter 4 :

Effect of chromium on seed germination and root development of *Sesbania cannabina* (Retz.) Poir. in two different growth media.

Abstract

Sediments of the Buriganga Riverbanks in Bangladesh are contaminated with heavy metals from industrial activity, especially chromium (Cr). *Sesbania cannabina,* a leguminous fodder crop with rapid growth and high biomass production, is found to be naturally growing in these areas and may have phytoremediation potential. This study aims to determine whether *S. cannabina* can be grown from seed under hexavalent chromium-stressed conditions to establish this species as a tool for phytoremediation. In this experiment, toxicity testing of hexavalent chromium ($K_2Cr_2O_7$) on seed germination was carried out using two growth media: Murashige and Skoog basal medium (MS); and Whatman Grade 1 filter paper (FP), by the top of media or top of paper method respectively. Under low Cr concentrations (≤ 50 ppm), no significant effect was observed in germination or root length. Under high Cr concentrations (increments ranging from 50 to 1000 ppm), 98-100% of seeds germinated in both growth media, but root length decreased to less than half the length of controls in ≥ 250 ppm Cr, and root elongation was negligible or stopped in ≥ 500 ppm Cr after 96h. Confocal micrographs (stained with propidium iodide) further indicate that damage to the cell wall of lateral root tips of germinated seeds increased with the concentration of Cr(VI), visible from ≥ 175 ppm. There was no significant difference observed between the two-growth media. Thus, it can be concluded from this study that *S. cannabina* can tolerate Cr contamination and can germinate and grow in concentrations of up to 175 ppm Cr(VI).

Keyword: hexavalent chromium; *Sesbania cannabina*; germination; root elongation.

4.1 Introduction

Many plant species can survive and even thrive in heavy metal-contaminated soil and may have phytoextraction capability depending on the element, metal speciation and plant species (Peralta-Videa et al., 2009; Xiong and Wang, 2005). These plant species may be exploited for decontaminating heavy metal-polluted soils as a low-cost and efficient alternative to existing physical or chemical decontamination processes that may damage the surrounding environment (Malik et al., 2010; Munir et al., 2010; Bah et al., 2010). Seed sprouting and root growth assays have been used to evaluate the heavy metal phyto-toxicity of hazardous waste sites (Salvatore et al., 2008; Kuriakose and Prasad, 2008) and help to understand the phytoremediation capability of the plants (Siddiqui et al., 2014). Seed germination is hindered or stopped due to HM in media (e.g. soil) (Ashraf et al., 2019; Shrestha et al., 2019), but the degrees of influence of heavy metal on germination depends upon plant species and metal types (Kranner and Colville, 2011). Response to HM during germination is one of the potential parameters for selecting the plant for phytoremediation studies. In contrast to research on the phytoremediation of different HM, studies on chromium phytoremediation have got less attention from plant scientists (Hayat et al., 2012; Srivastava et al., 2021).

Many plants show wide tolerance to Cr-contaminated environments (López-Luna et al., 2009). Chromium toxicity to seed germination is the first physiological effect of Cr on plants (Singh et al., 2013). Seed germination in a Cr-enriched environment depends on the plant's ability to withstand Cr toxicity. For example, seeds of *Avena sativa* showed 84% germination inhibition while grown in 4000 ppm Cr(III) (López-Luna et al., 2009), *Hibiscus esculentus* seeds showed 90% germination inhibition

for 100 ppm Cr(VI) (Amin et al., 2013) and *Medicago sativa* seeds showed 23% germination inhibition only for 40 ppm Cr(VI) (Peralta et al., 2001).

Scientists worldwide are continuously trying to find a suitable plant for phytoremediation of Cr(VI) contaminated soil and sediment. *Sesbania cannabina,* a fast-growing leguminous plant, might be an appropriate candidate as a tool for phytoremediation for Cr(VI) contaminated soil or sediment (Sarwar et al., 2015; Iqbal et al., 2019; Ali et al., 2013). However, knowledge gaps exist in the response of *Sesbania cannabina* to the toxicity of hexavalent chromium. The aim of this study was to determine whether Cr(VI) contamination affects *S. cannabina* germination and root growth as an indicator of suitability for use in a phytoremediation study.

4.2 Methods

For use in all experiments, seeds of *S. cannabina* were collected from Shobuz Biz Bhar, Bangladesh, a locally reputed seed-selling company and stored under dry conditions at room temperature until use.

4.2.1 Experimental design

Assessment of toxicity of chromium ($K_2Cr_2O_7$, ICP grade) on seed germination of *S. cannabina* was carried out using the top of the paper (Whatman® 1 filter paper (FP)) and top of media method (modified Murashige and Skoog basal medium (MS)) (Siddiqui et al., 2014; Healthcare, n.d.). In all cases, seeds of *S. cannabina* were pre-treated with H_2O_2 (6% v/v) for 5 minutes and primed with 65°C water for 5 minutes before adding Cr(VI) in Petri dishes. Germination chamber conditions were 12 h full spectrum light at temperature 28°C± 1°C and relative humidity of ~

75% for 5 days. This experiment recorded ≥ 2mm radicle protuberance from the seed's testa as evidence of germination (Adhikari et al., 2022; Vidak et al., 2022). Seed germination percentages were measured based on the total germination count of each replicate 5 days after incubation. A range of concentrations of Cr(VI) (as $K_2Cr_2O_7$) (0, 5, 10, 25, 50, 100, 175, 250, 500, 750 and 1000 ppm) was added to the two different types of media in Petri dishes to observe the effect of Cr(VI) on seed germination. Each petri dish contained 10 sterilised (pre-treated) seeds, and six replicates were prepared for each treatment medium.

4.2.2 Seed selection, preparation and growth media for germination

Seed selection was performed by examination under a dissecting microscope, and deteriorated seeds (e.g., dirty or void seeds) were rejected. Healthy seeds were soaked and sterilized (orbital shaker, 200 RPM, 5 minutes) with hydrogen peroxide (6% v/v) and rinsed with running deionized water for 5 min to avoid microbial contamination. This was followed by 65°C±1°C hot water treatment for 5 min to achieve 99±1% germination. After treatment, seeds which appeared to be fully imbibed were selected for investigation.

In this experiment, six replicates of each dose containing 10 seeds were evenly placed in a 9-cm-diameter petri dish on growth medium (double layer of Whatman No. 1 filter paper (FP) and Murashige and Skoog basal medium (MS)). The growth media were then moistened with 5.0 ml of Cr(VI) solution, the Petri dishes were wrapped with para-film to avoid evaporation and placed in a vitopod propagator (temperature controlled, fixed at 28°C±1°C) under full spectrum light.

4.2.3 Germination percentage

Germination percentage (at 12-hour intervals) was calculated using the formula (Bae et al 2016):

$$\text{Germination percentage, } TG_{hour} = \frac{\text{Number of germinated seeds at fixed hour}}{\text{Total number of seeds}} \times 100$$

4.2.4 Germination Rate (T_{50})

The number of germinated seeds was recorded every 12 h for 5 days. Germination rate (T_{50}) refers to the time (hours) to reach 50% of final germination over the 5 days trial and was calculated using the formula (Farooq et al., 2005):

$$T_{50} = \frac{\{(\mathrm{N}/2) - \mathrm{ni}\}(\mathrm{ti} - \mathrm{tj})}{(\mathrm{ni} - \mathrm{nj})}$$

Where N is the final number of seed germination at 5 days time (or 120 h) and ni, nj is the cumulative number of seeds germinated by consecutive counts at times ti and tj measured in hours when ni < N/2 < nj.

4.2.5 Radicle (RTI) tolerance indices

The radicle length of germinated seeds was measured from the radicle shoot junction to the tip of the longest radicles after 5 days or at 120 h and expressed as RTI using the formula (Guterresa et al., 2019; Shafiq and Iqbal, 2006):

$$\mathrm{RTI} = \frac{\text{Length of the longest radicle in metal treatment}}{\text{Length of the longest radicle in sterilized deionized water}} \times 100$$

4.2.6 Confocal microscopy

To observe the effect of Cr(VI) on *S. cannabina* cell structure of the seed radicle, segments of roots 1.5-2 mm in size were taken and stained with 1 µg/ml Propidium

Iodide (PI) after 120 h. Red fluorescence was then observed under the confocal microscope in the cell wall to determine cell damage (Shi et al., 2016). In healthy cells, the stain remains at the perimeter of the cell. In disrupted cells, the dye enters the cell, giving a red cell body and nucleus. PI was excited at 488 nm, and fluorescence was detected at ≥ 585 nm (red channel) (Coskun et al., 2012) using a Zeiss LSM710 ConcoCor3 confocal microscope (Carl Zeiss, Jena, Germany).

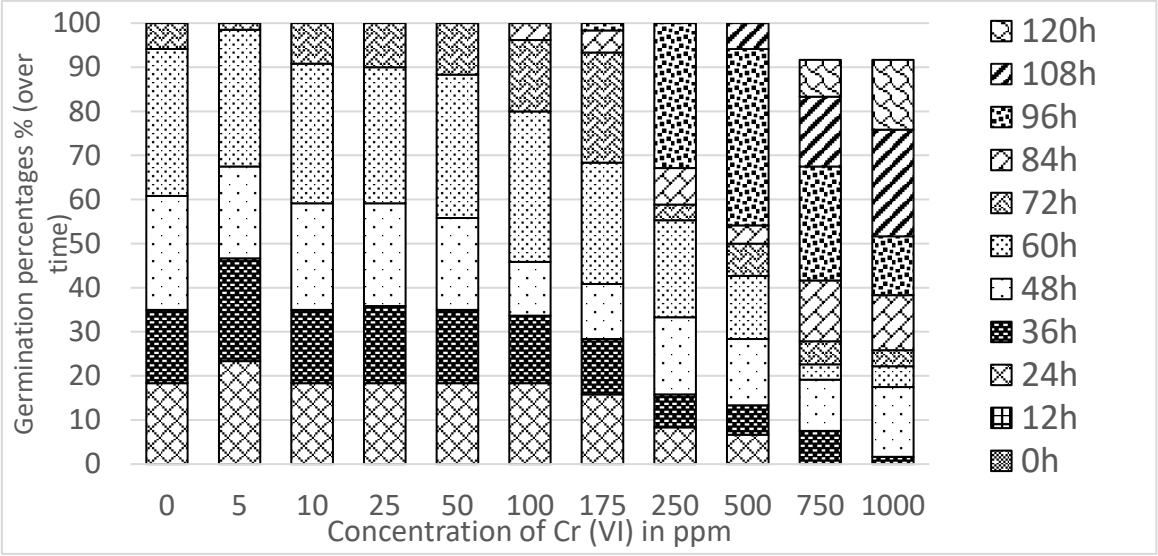
4.2.7 Statistical analysis

The One-way analysis of variance (ANOVA) was carried out with SPSS (v 25) to determine significant differences among treatments and we checked normality and homogeneity of variance assumptions before ANOVA. When a significant ((p ≤ 0.001)) difference was observed between treatments, multiple comparisons were made by the Tukey test. A Pearson's correlation analysis was carried out to establish the relationships between phytotoxicity and Cr(VI) dose.

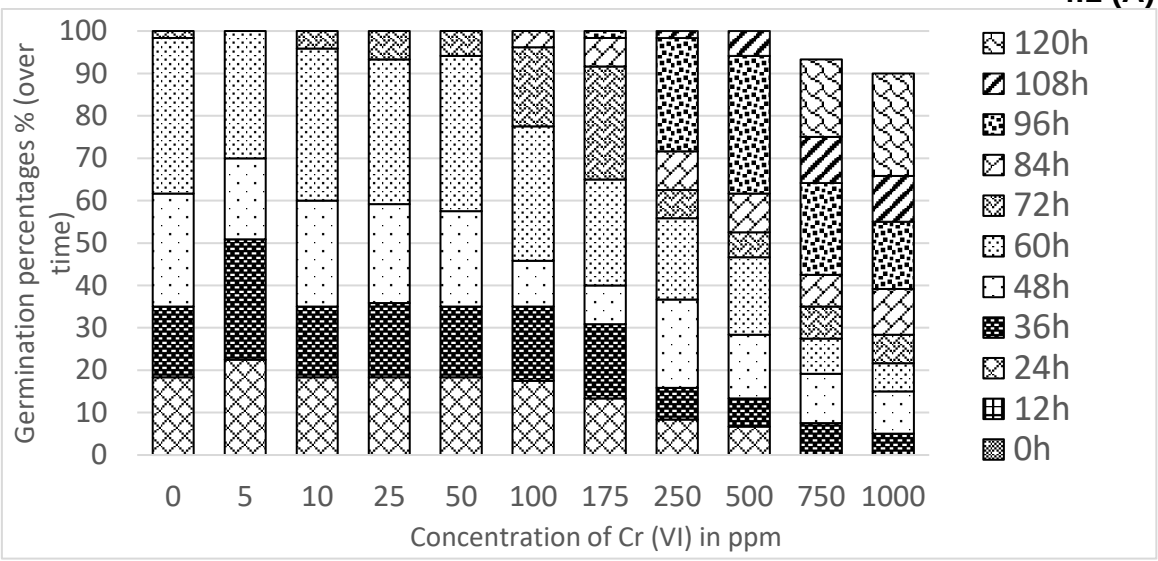
4.3 Results

4.3.1 Seed germination

In this study, during exposure to different concentrations of Cr(VI) (0, 5, 10, 25, 50, 100, 175, 250, 500, 750 and 1000 ppm), concentrations ≤ 100 ppm were observed to have no significant effect (p >0.05) on final seed germination percentage for both growth media (figure 4.1) at ≥ 72 hours, but germination significantly decreased at concentrations ≥ 175 ppm (p ≤ 0.001). Indeed, in increasing Cr concentrations up to 100 ppm in both growth media (MS and FP), the seed germination percentage was almost constant (98±2 %, Figure 4.2) at 72 hours.



4.1 (A)



4.1 (B)

Figure 4.1: Germination percentage of *S. cannabina* recorded at 12-hour intervals during exposure to different concentrations of Cr(VI), shown in 4.1 (A) with MS0, 4.1 (B) with FP (n=60). MS refers to germination on modified Murashige and Skoog basal medium, and FP refers to germination on Whatman No 1 Filter paper.

In addition, there was no statistically significant difference observed among the germination percentages of the controls (0 ppm) and Cr-treated seeds at 48 hours up to 175 ppm, but there was some delay in germination percentage observed with increasing Cr concentration at 36 h compared to seed germination at 5 ppm (figure 4.1(A) and 1(B)). However, some stimulation was observed at 5 ppm for both MS

and FP medium (figure 4.1 (A) and 4.1 (B)). A significant difference in germination percentage at 36 hours was also found for seeds exposed to 100 to 1000 ppm Cr(VI). However, seed germination percentages were not significantly different from each other as detected by Tukey's LSD ($p \leq 0.001$) for 0 ppm, 10 ppm, 25ppm, 50 ppm and 100 ppm, but germination percentages at 5 ppm showed a significant difference ($p \leq 0.001$) with all other treatments.

For concentrations of Cr ≥ 175 ppm seed germination percentage decreased over time. After the seed imbibition, 95±5 % germination was observed at various times for each treatment; 96h for 250 ppm, 108 h for 500 ppm and unable to germinate 100 % at 750 ppm and 1000 ppm within 120h for both media (figure 1(A) and (B)). Compared to the control, germination percentage decreased (except 5 ppm) with increased Cr concentration at all sample times. A strong negative correlation ($r=-0.994$, $p < 0.05$) has been recorded for the final germination percentage (for 175, 250, 500, 750 and 1000 ppm at 120 h) with chromium concentration.

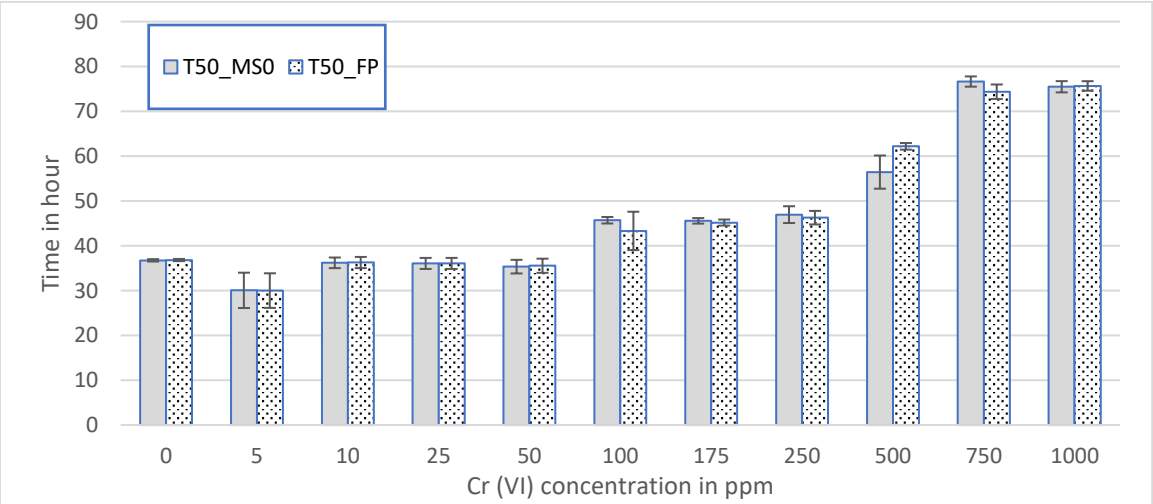
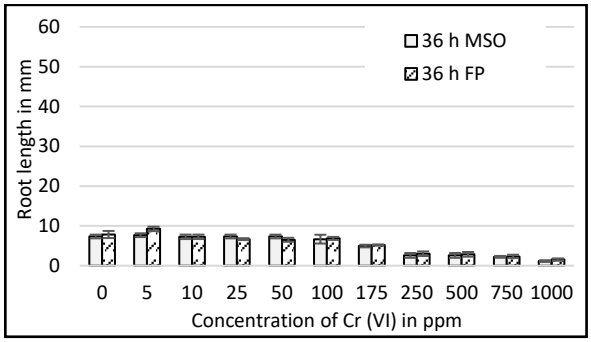


Figure 4.2: Germination Rate (T_{50}) of *S. cannabina* at different concentrations of Cr. Error bars are standard deviation from the mean (n=60). MS refers to germination on modified Murashige, and Skoog basal medium and FP refers to germination on the Whatman No 1 Filter paper.

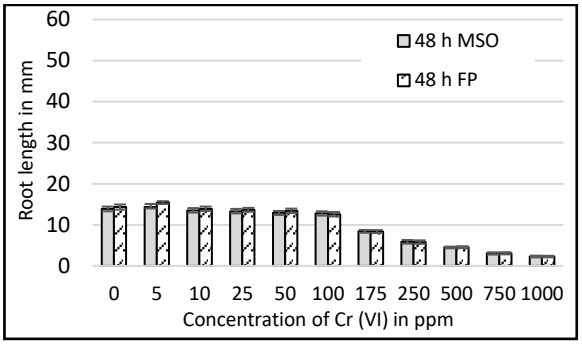
In this experiment, the germination rate (T_{50}) increased ($p \leq 0.01$) as the concentration increased from ≥ 100 ppm (figure 4.2). ANOVA results showed that there were no significant changes in seed germination rate (T_{50}) at 0ppm, 10 ppm, 25ppm, and 50 ppm, but T50 at 5 ppm showed a significant difference ($p \leq 0.001$) in germination rate (T_{50}) and also showed a substantial increase in T_{50} with an increase of Cr(VI) concentration ≥ 100 ppm dose (figure 4.2).

4.3.2 Maximum Root elongation

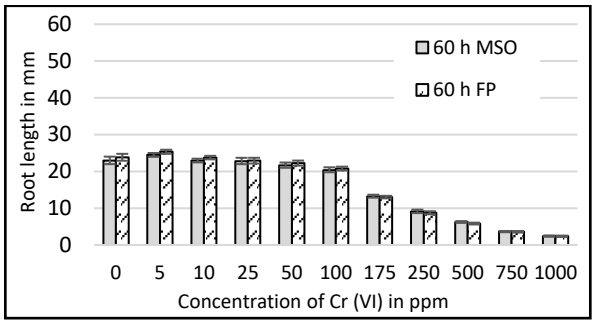
A one-way ANOVA was conducted to compare the effect of Cr(VI) concentration on root elongation for different Cr(VI) concentrations at 120h. There is an apparent decrease ($p \leq 0.001$) in average root length with an increase in Cr(VI) over time (figure 4.3); however, this was due to delayed seed germination or destruction of root cells with increasing Cr concentration. On the contrary, stimulation of germination was observed at 5 ppm Cr(VI) (figure 4.3(H)) with a higher root length relative to controls for both mediums ($p < 0.001$ for MS0 and $p < 0.001$ for FP). Changes in root growth over time were not significantly different from each other as detected by Tukey's LSD ($p \leq 0.001$) for 0 ppm, 10 ppm 25 ppm and 50 ppm concentrations at 120h. However, there was a significant negative effect ($p<0.05$) (at 120 h) of Cr(VI) concentration on root elongation for 100 ppm, 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. Root length decreased with an increase in Cr concentration ≥ 100 ppm in both media (figure 4.3, 4.3(C) and 4.3(D)). Root length at 250 ppm was less than half (32.15 % for MS0 and 31.59 % FP) and at 500 ppm was less than a quarter (21.48 % for MS0 and 31.59 % FP) compared to controls at 120 h. At higher concentrations ≥ 750 ppm, root elongation was less than 5 mm for both media at 120h.



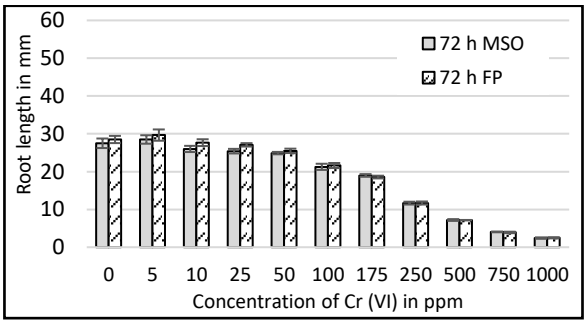
4.**3 (A)**



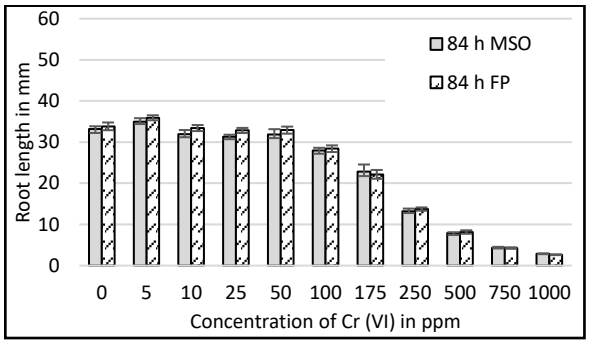
4.**3 (B)**



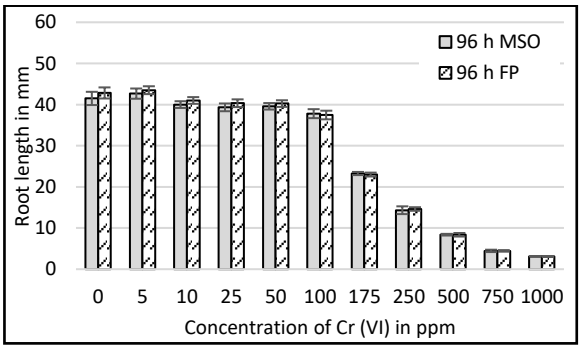
4.**3 (C)**



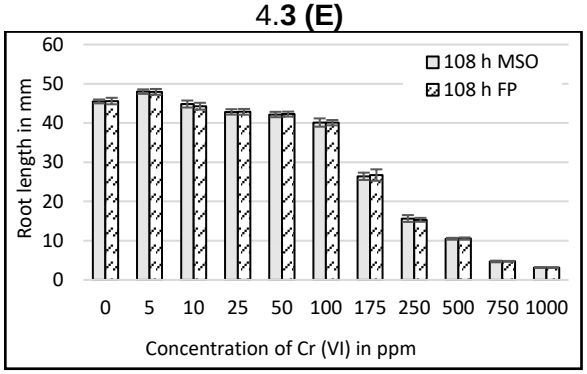
4.**3 (D)**



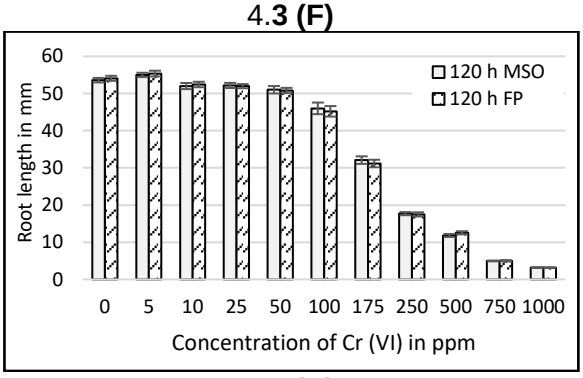
4.**3 (E)**



4.**3 (F)**



4.**3 (G)**



4.**3 (H)**

Figure 4.3: Effect of Chromium on early seedling root length (mm) of *S. cannabina* recorded at 12-hour intervals during exposure to Cr(VI) in both mediums. 4.3 (A) 36h, 4.3 4. (B) 48h, 4.3 (C) 60h, 4.3 (D) 72h, 4.3 (E) 84h, 4.3 (F) 96h, 4.3 (G) 108h and 4.3 (H) 120 h shows *S. cannabina* grown in different concentration of Cr(VI) in both medium. Error bars are standard deviation from mean (n=6). MS0 = Murashige and Skoog basal medium and FP = Whatman No 1 Filter paper (average of highest root length of each treatment calculated and plotted against concentration over time).

A strong negative correlation was observed between Cr(VI) concentration and radicle (RTI) tolerance indices (table 1) (r = - 0.942, n = 66, p= < 0.001 for MS0 and r = - 0.948, n = 66, p= < 0.001 for FP) for both MS and FP. From the correlation results, it is clear that root length decreases as Cr(VI) increases, and there was no significant difference observed between the two different germination media.

Table 4.1: Radicle (RTI) tolerance indices (%) after 5 day or 120h for different Cr(VI) concentrations (0 to 1000 ppm).

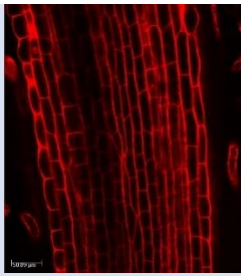
	MS0	FP
0 ppm	100± 0[a]	100.00±0 [a]
5 ppm	102.65± 0.58[b]	102.34±0.77[b]
10 ppm	97.05± 3.82 [a]	96.92±3.73 [a]
25 ppm	97.36± 2.69 [a]	96.09±3.55 [a]
50 ppm	95.18± 4.00 [a]	93.93±5.72 [a]
100 ppm	85.85± 1.56[c]	83.57±2.42[c]
175 ppm	59.88± 1.02[d]	57.63±2.02[d]
250 ppm	33.00± 0.42[e]	32.33±2.56[e]
500 ppm	22.05± 0.40[f]	23.17±3.42[f]
750 ppm	9.24± 0.10[g]	9.24±2.18[g]
1000 ppm	5.91±0.09[h]	5.79±1.07[h]

**Results are given as the mean and standard error of six. Treatments with the same letter in same column shows no significant difference (P > 0.05) from the control (0 ppm).

There was no significant difference between RTI up to 50 ppm concentration. As the chromium concentration increased ≥ 100 ppm , the RTI decreased significantly (p ≤0.001) and reached 5.76% for MS0 and 5.66% for FP compared to the control value at 1000 ppm (table 4.1).

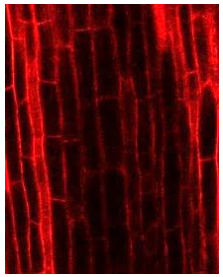
4.3.3 Effect of Cr(VI) on root structure

Figure 4.4 shows confocal micrographs of PI staining of the cell walls. In the controls (Cr = 0 ppm), fluorescence was localised to the cell wall, as cell walls are not weakened, and the PI cannot enter the cell (figure 4.4, A1 and A2). The cell wall remained intact up to 50 ppm Cr(VI) (not shown in the figure) but started to disrupt at ≥ 100 ppm. At 100 ppm concentration, ≤ 2 % cells were disrupted (figure 4.4 (B1) and 4.4 (B2).



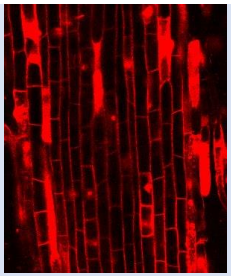
0 ppm Cr in MS0

4.A 1



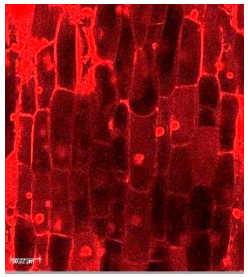
100 ppm Cr in MS0

4.B 1



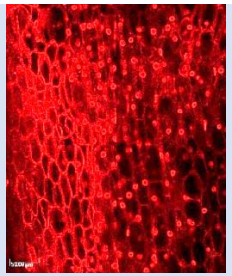
175 ppm Cr in MS0

4.C 1



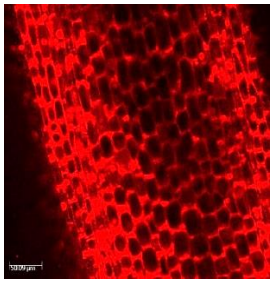
250 ppm Cr in MS0

4.D 1



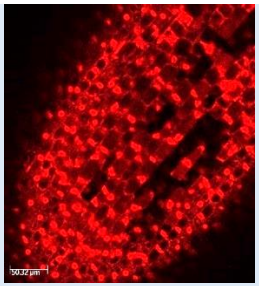
500 ppm Cr in MS0

4.E 1



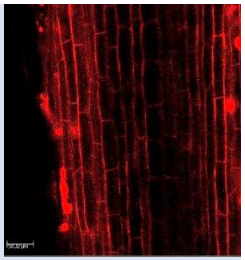
750 ppm Cr in MS0

4.F 1



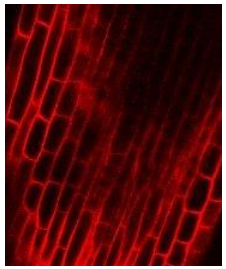
1000 ppm Cr in MS0

4.G 1



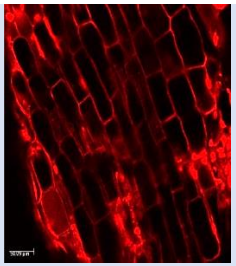
0 ppm Cr in FP

4.A 2



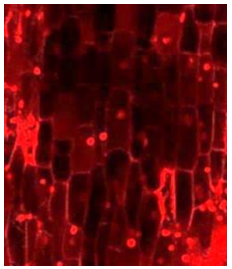
100 ppm Cr in FP

4.B 2



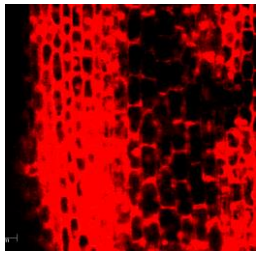
175 ppm Cr in FP

4.C 2



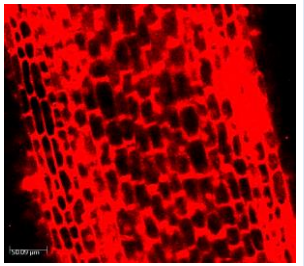
250 ppm Cr in FP

4.D 2



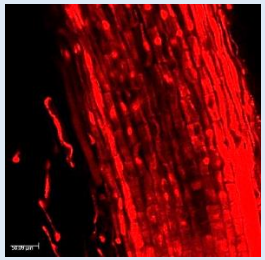
500 ppm Cr in FP

4.E 2



750 ppm Cr in FP

4.F 2



1000 ppm Cr in FP

4.G 2

Figure 4.4: Confocal micrographs (stained with propidium iodide) of the cell wall and damaged cell nuclei (lateral root tips) of *Sesbania cannabina* after 5 day or 120 hours in (A1, A2) 0 ppm (control), (B1, B2) 100 ppm, (C1, C2) 175 ppm; (D1, D2) 250 ppm; (E1, E2) 500 ppm, (F1, F2) 750 ppm and. Scale bars represent 50 µm ± 1 µm. MS0 = Murashige and Skoog basal medium and FP = Whatman No 1 Filter paper.

At 175 ppm cell damage started and fluorescence intensity increased. As the concentration of Cr in the media increased (≥ 250 ppm), more cells with stains internal to the cell were observed, and red nuclei showed that increased Cr increased damage to the cells of root tips (figure 4.4, D1 and 4. D2). Cell shape also gives an indicator as to the effects of Cr on root cell growth. Compared to the control, the samples at 250 ppm Cr concentration showed a higher number of damaged cells, but the shape of cell wall remained the same. More damaged cells were observed for ≥ 500 ppm concentration (figure 4.5 (E1) and (E2). Complete cell damage, shape distortion and shrinkage of the cell wall was observed for root cells grown in ≥ 750 ppm (figure 4.4 (F1) and (F2)) and 1000 ppm (figure 4.4 (G1) and (G2)).

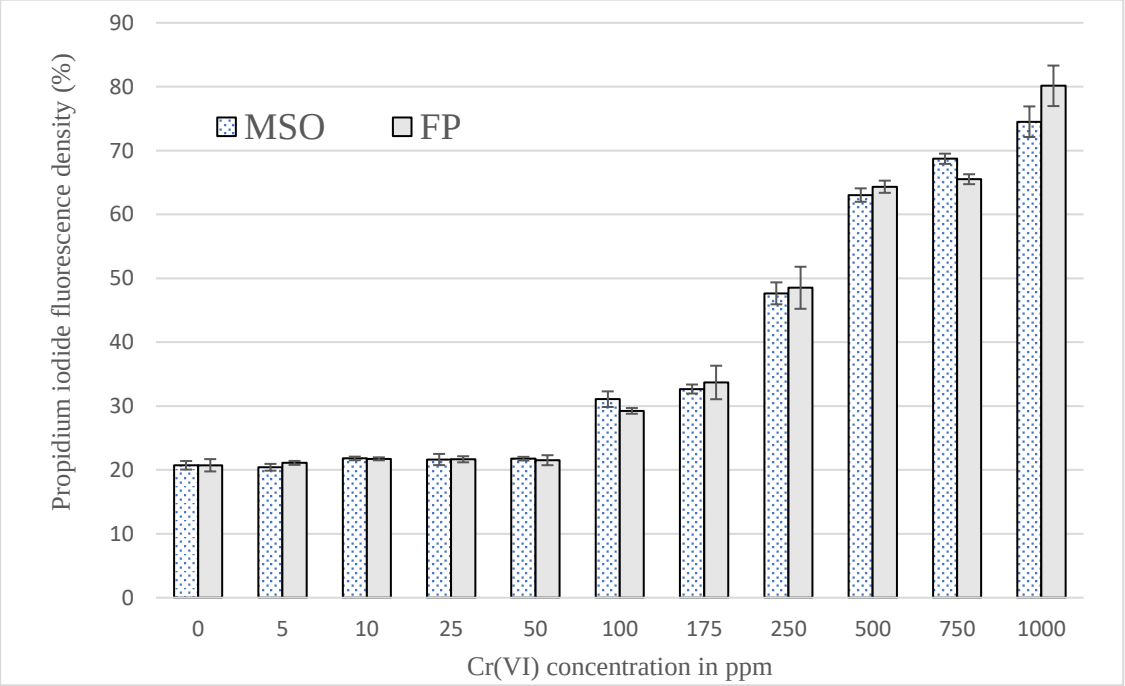


Figure 4.5: Propidium iodide fluorescence density in the roots grown in different concentration of Cr(VI) (0, 5, 10, 25, 50, 175, 250, 500, 750 and 1000 ppm) after 120 h . MS0 = Murashige and Skoog basal medium and FP = Whatman No 1 Filter paper. Error bars are standard deviation from the mean (n=3).

To quantify cell damage, Image J® (fluorescence density analysis) suggests that fluorescence intensity increases with the increase of hexavalent chromium in the medium (Shi et al., 2016; Ortega-Villasante et al., 2005). The fluorescence intensity (dead cells or cell wall) in the elongation zone of roots (exposed to different concentrations of Cr(VI) from the beginning of the germination) after 120 h was assessed and found not significantly different from each other as detected by Tukey's LSD ($p \leq 0.001$) for 0 ppm, 5 ppm, 10 ppm, 25 ppm and 50 ppm but significantly increases in fluorescent intensity≥ 100 ppm at 120h ($p \leq 0.001$). The present study strongly suggests that with the increase of chromium concentration, cell shape changes by shrinking, distortion and cell wall rupturing, shown by stronger fluorescence inside the cell body as Cr concentration increases from 100 ppm to 1000 ppm.

4.4 Discussion

Hexavalent chromium (as $K_2Cr_2O_7$) tolerances were tested by allowing seeds to be germinated in a metal-containing medium because germination is the initial physiological development process altered by the metal (Peralta et al., 2001). Metal toxicity assessment in plants has been assessed by seed germination and root elongation (Salvatore et al., 2008; Cheng and Zhou, 2002). In the present study, the two parameters (seed germination and root elongation), along with the fluorescence confocal micrograph, were measured and calculated in response to Cr(VI) for two separate media.

At 5 ppm concentration, we observed a stimulation effect on seed germination percentage (and root length with time) compared to control (0 ppm) in both media, and the T_{50} (figure 4.2) germination rate also indicated the lowest time required for

T_{50} (30.05 h for MS0 and 30.001 h for FP). This result is supported by the work carried out by Dixit et al., (2002), who observed low concentration of Cr(VI) ≤ 2.5 ppm increases germination (and root growth) in *Pisum sativum* L. cv. Azad (Pea plant) compare to control. Again, Peralta et al., (2001) also reported a stimulating effect on root elongation compared to the control for *Medicago sativa* L. (Alfalfa) at 5 ppm Cr(VI) treatment. Some other work has found low concentrations of heavy metals (cadmium, arsenic and copper) to have a minor stimulation effect on seed germination percentage (Lefèvre et al., 2009; Kjær et al., 1998; Chun-xi et al., 2007). It has been well-studied that chromium is a non-essential element for plant growth and development (Hayat et al., 2012; Wakeel and Xu, 2020), but according to Lefèvre et al., (2009), this slight stimulation in germination percentage is due to a low concentration of HM because HM causes oxidative stress (Vardhan et al., 2019), which encounters the reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Tsukagoshi, 2016). In this study, we observed the highest root elongation for 5 ppm treatment at any time, and RTI results (table 4.1) also suggest this.

Notwithstanding the enhanced seed germination at 5 ppm Cr, chromium doses up to 100 ppm had no measurable effect on seed germination percentage ≤ 100 ppm Cr(VI) at ≥ 72h. This may be because seed coats create a barrier between the embryo and the surrounding environment during and after the imbibition and absorb low amounts of Cr(VI) at a low level of contamination (below 100 ppm), thus preventing harmful effects of contaminants on embryonic root growth (Araújo and Monteiro, 2005; Akinci and Akinci, 2010). T_{50} results (figure 4.2) suggest that no significant changes were observed in T_{50} at ≤ 50 ppm, but significant differences were observed in T50 results at ≥ 100 ppm. The root elongation result (figure 4.3)

suggests that for 100 ppm treatment, there was a significant delay in root elongation compared to control and RTI at 120 h (83.64 % for MS0 and 81.66 % for FP) also shows decrease in RTI. Dixit et al., (2002) reported in *Pisum sativum* L. cv Azad (pea) approximately 15± 2% reduction in root length (at 20.7 ppm Cr(VI)) compared to control because Cr(VI) induced oxidative stress disrupted mitochondrial electron transport. The study of root elongation is considered more insightful and more responsive than germination studies for understanding the toxicity of heavy metals (Hou et al., 2014; Araújo and Monteiro, 2005). This is because, compared to other plant tissues, roots are directly exposed to heavy metals, and it is the initial organ that absorbs the nutrients from the soil (Hou et al., 2014).

At Cr(VI) concentration ≥ 175 ppm, strong negative correlations have been observed between chromium concentration and germination percentages because during the imbibition, seed cover softens and becomes more vulnerable (permeable) to numerous stresses (Kranner and Colville, 2011; Wierzbicka and Obidzińska, 1998). This has been suggested to be linked to disruption in oxygen utilization and utilisation or mobilisation of the reserve food stored in seed (Seneviratne et al., 2017). High fluorescence density was observed in the confocal micrograph (at 175 ppm) compared to the control, and greater cell damage was observed (figure 4.4 C (1) and 4.4(C2)). Despite the cell damage and decrease in root length, the *S. cannabina* seeds can germinate, and root length continuously increases over time, thus indicating the capability of the seeds are able to germinate.

In a similar study, Sahoo et al., (2018) examined the effect of hexavalent chromium (up to 80 ppm) on germination and growth of *Sesbania cannabina*,. They reported germination percentages for 80 ppm is about 27.5± 1.5 %, but germination at 0 ppm

was only 57.5± 0.5 % because they did not follow seed selection protocol (health seed) or prime the seeds with hot water (or other treatment) to achieve 99±1 %. Most importantly, they observed radicle lengths of 41 mm at 20 ppm and 19.5 mm at 80 ppm, but they did not consider the standard size for radicles. For example, the standard growth of seed radical size is ≥ 2mm considered as germinated (Liu et al., 2003, 1994). It is therefore, essential to select and prime seed to ensure the maximum germination at control for the credibility of the seeds for germination study. Indeed, to observe the detrimental effects of contaminants (heavy metal), researchers should use a higher concentration of contaminants to study seed germination percentages because the study will give a clear insight of the heavy metal effect on seed germination to understand their capability to grow in wide concentration of contaminant (Peralta et al., 2001; Munzuroglu and Geckil, 2002). In this study, after 120 hours at ≥ 250 ppm (250 ppm, 500ppm, 750 ppm and 1000 ppm) concentration, ≥ 95% (for both mediums) germination was observed for both media, but a significant reduction in root length was observed for 250 ppm, and 500 ppm Cr(VI) concentration and root elongation stopped ≥ 750 ppm (figure 4.3). Heavy cell damage was observed with the confocal microscope (figure 4 E (1) and E(2)) at 500 ppm. We considered the germination standard in this experiment when the root length (cell division) through the seed coat was ≥ 2mm. However, root length is not always represented by cell division (Liu et al., 2003, 1994) but may be due to cell elongation (Chon et al., 2004; Haber and Luippold, 1960). It has been reported that chromium (VI) accumulates in root cells resulting in the hinderance of plants' radicle growth (Hou et al., 2014). Chromium immobilises in vacuoles of the root cell, helping Cr accumulate in the root, causing wilting and plasmolysis in root cells (Shanker et al., 2005). Figure 4.4 shows almost all outer cells of the root were destroyed at ≥

250 ppm of Cr(VI), and fluorescent density (figure 4.5) results also indicate the findings. This suggests that ≥ 250 ppm Cr(VI) concentration severely hindered germination in both media, and thus *S.cannabina* is unable to grow ≥ 250 ppm Cr(VI) contaminated soil. Indeed, our confocal microscope images (figure 4.4B (1) and 4B (2)) detected disruption of root cells from 175 ppm. The seeds radicle of *S. cannabina* are able to grow upto 2 mm (indicates germination) up to 1000 ppm Cr but unable to grow (root elongation) ≥ 250 ppm Cr(VI), thus indicating that the plant might be used as a phytoremediator in conditions of ≤ 175 ppm Cr(VI).

4.5 Conclusion

The toxicity of hexavalent chromium depends on various factors. In this study, we assessed the Cr(VI) concentration in which seeds of *S.cannabina* can germinate and grow to provide an indication of the potential for the plant's phyto-management capability for Cr(VI) contaminated land or sediment. Considering the germination percentage results, root elongation study and confocal images, *S. cannabina* seeds can grow up to 175 ppm of Cr(VI) concentration and root growth stopped ≥ 250 ppm. Germination and root growth results suggest the species is a good candidate for the phytoremediation of Cr(VI) concentrations up to 175 ppm Cr(VI).

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Chapter 5 :

Evaluation of the root system of *Sesbania cannabina* (Retz.) Poir. grown in hexavalent chromium contaminated soils by utilising modified rhizobox systems.

Abstract

Hexavalent chromium is one of the most toxic heavy metals for plants and animals. This poisonous metal contaminates the soil via anthropogenic activities. This study aimed to assess how the root system of *Sesbania cannabina* behaves under various concentrations of Cr(VI) and whether it could be a suitable species for the phytoremediation of Cr(VI) contaminated soils. The experiment was conducted in rhizoboxes under greenhouse conditions using a sandy loam soil dosed with potassium dichromate giving eight different Cr(VI) concentrations (0 ppm, 5 ppm, 10 ppm, 20 ppm, 40 ppm, 80 ppm, 160 ppm, and 360 ppm). Plant roots were photographed with a Canon 60D (18-megapixel) camera with a 50 mm prime lens and analysed with Image J image processing software. Cr(VI) in samples were prepared by alkaline digestion method and analysed with a UV-visible spectrometer at a wavelength of 540 nm. At 360 ppm concentration, seeds of *S. cannabina* germinated but were unable to grow further. However, under 0-80 ppm concentrations, no significant change was observed in the root growth (length). At 160 ppm, root growth was reduced by about 55±0.65% at 25 days and 35±0.25 % at 45 days compared to plants grown at 0 ppm. After 45 days, no chromium (VI) was detected in the soil for (0 to 160 ppm); however, for the controls (0 to 160 ppm with no plants), we observed no changes in Cr(VI). The absence of Cr(VI) in the soil after 45 days suggests that *S. cannabina* can be considered a candidate for phytoremediation of soils containing up to 160 ppm Cr(VI).

Keywords: *Sesbania cannabina,* hexavalent chromium, rhizobox, root.

5.1 Introduction

Anthropogenic activities that increase heavy metal pollution in soil and water have become a major environmental and health problem worldwide (Fu and Wang, 2011; Li et al., 2019; Vardhan et al., 2019). Soil fertility and crop production decrease due to heavy metal deposition in soil, whilst heavy metals may also accumulate in crops and enter the food chain (Grant, 2011; Zwolak et al., 2019). Hexavalent chromium [(Cr(VI)] is one of the most carcinogenic heavy metals which contaminates the environment mostly (especially soil) through anthropogenic activities such as mining, tanning and other industrial activities (Li et al., 2019; Zwolak et al., 2019).

Several methods have been established for the remediation of contaminated soil (Liu et al., 2012; Molina et al., 2013; Yi et al., 2011). Biological remediation methods are more eco-friendly and less expensive than chemical and physical remediation methods for contaminated soil treatment (Ashraf et al., 2019; Grzegórska et al., 2020). Researchers worldwide are trying to find a suitable species for the phytoremediation of different pollutants because these methods are considered the cheapest and more environmentally friendly than conventional methods (Ashraf et al., 2019; Grzegórska et al., 2020).

In the phytoremediation technique (especially for soil), roots first come into contact with contaminants in the rhizosphere; a complex zone where roots and microorganisms interact with soil and interstitial water. The roots release hydrogen ions and metabolites (exudates) into the soil, thus changing the soil pH and altering nutrient uptake capacity (Marschner and Römheld, 1983; Lin et al., 2004). Root exudates are low molecular weight organics of the root that play a vital role in nutrient uptake and detoxification of hazardous substances (Jones, 1998; Ryan and Delhaize, 2001). Root exudates and microbes in the rhizosphere can alter the

speciation of heavy metals before they interact with the plant root system (Lynch and Leij, 2012; Pinel et al., 2003; Waldrip et al., 2011). In addition to that, root exudates change the dissolved organic carbon (DOC) in soil (Villarino et al., 2021; Ma et al., 2022).

Plants have the capacity to modify root structure to increase the nutrient uptake capacity in stressed conditions (Funakoshi et al., 2018). The study of RSA (root system architecture) has become a promising tool for determining the changes in root structure in response to variations in nutrients and contaminant soil concentration (Giehl and von Wirén, 2014; Voss-Fels et al., 2018). A rhizobox (microcosm) study, an easy, fast and non-destructive method, is widely used by plant scientists to uncover and visualise the active root zone (Neumann et al., 2009; Jia et al., 2019; Corzo Remigio et al., 2021). This rhizobox consists of one observation window made with a transparent polymer sheet (PVC, polyvinyl chloride) panel for root imaging (figure: 2.1) (Lesmes-Vesga et al., 2022; Corzo Remigio et al., 2021; Soledad Graziani et al., 2016). The rhizoboxes do not affect root growth and nutrient uptake; thus, plants grown in rhizoboxes can be compared with naturally growing plants (comparisons among species) (Mašková and Klimeš, 2020).

In this experiment, we have selected a high-yield leguminous fodder crop, *Sesbania cannabina,* to observe the effect of Cr(VI) in the root system (Sarwar et al., 2015). This plant can be found in semi-arid to sub-humid climates and can tolerate various environmental conditions, including seasonally submerged soils, and is common in Bangladesh, where Cr(VI) contamination is a problem (Ren et al., 2019; Sarwar et al., 2015). The study's main objective is to assess the changes in root system

architecture (RSA) under different concentrations of Cr(VI) of the *S. cannabina* using rhizoboxes.

5.2 Methodology

5.2.1 Soil Preparation

The soil used in the experiments was a sandy loam from Woburn® (UK national grid reference SP9736), which is described in Table 1. Air-dried soil was passed through a 2 mm sieve to maintain the homogeneity of the soil. The air-dry soil was subsequently enriched with Potassium-di-chromate ($K_2Cr_2O_7$) (ICP grade, Mark). Eight (including control) Chromium-containing soil concentrations were prepared (0 ppm, 5 ppm, 10 ppm, 20 ppm, 40 ppm, 80 ppm, 160 ppm and 360 ppm) and transferred to rhizobox.

Table 5.1: Parameters of soil used in this experiment (average values ± SD (n=3).

pH	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})	OM (%)	Sand (%)	Zn (ppm)	Cu (ppm)	Ni (ppm)	Cr (total) (ppm)	Cr(VI) (ppm)	Pb (ppm)
6.7-7	62± 2	87± 3	412± 8	3.1 to 3.3	60 to 61%	110± 2.4	36±1 .2	20 ± 1.1	9± 1.2	< 0.1	5±0.2 1

5.2.2 Seed germination, transfer to rhizobox and experiment design:

Seeds were germinated under controlled conditions; 12 h full spectrum light at temperature 28°C ± 1°C and relative humidity of ~ 75% for 3 days. Seeds were pre-treated with H_2O_2 (6% v/v) for 5 minutes and primed with 65°C water for 5 minutes. After 72 hours, three seeds with radicle sizes between 2mm - 4mm were transferred to the top of the soil and covered with 5mm of soil. The experiment design is discussed below

Step 1 Pre-treated seeds (see chapter 3) were allowed to germinate for 72 hours.

Step 2 Seeds with 2-5 mm roots radical were transferred to the rhizobox.

Step 3 Rhizobox photographed every 5 days (from the day of seed transfer).

Step 4 Plant (root, shoot and leaves) and soil samples collected after 45 days.

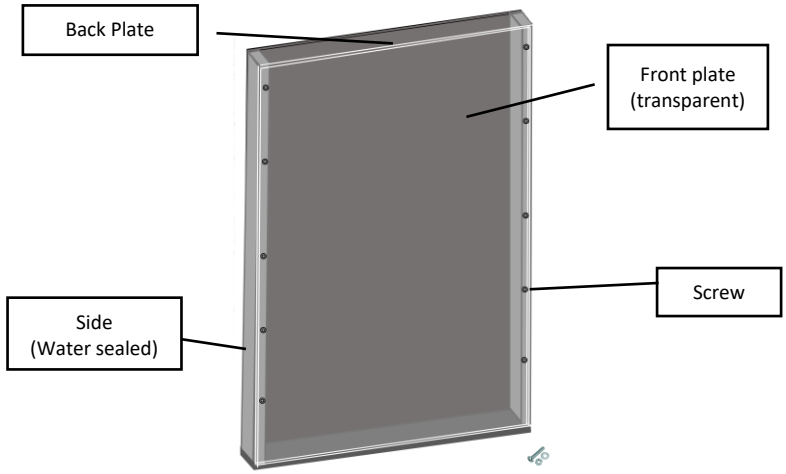
Step 5 Root wet weight and dry weight (dried at 80 °C to constant dry mass) determined.

Step 6 Plant and soil samples analysed for chromium (VI) (see section 5.2.6).

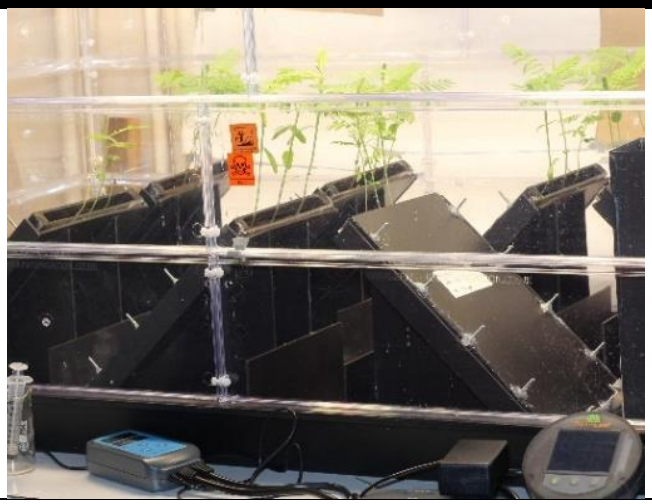
5.2.3 Rhizobox design and development

The design of the box is shown in figure 5.1 [.A Rhizobox; a transparent front plate (Acrylic Sheet, 3mm thickness) and a non-transparent back plate (Foamax® hard, 3mm thickness)].Figure 5.1B rhizobox in a laboratory growth chamber under artificial light (1000W LED Grow Light, MIVARRS® 2x2 ft Plant Grow Lights Full Spectrum) with temperature and humidity controls. The boxes were placed inclined at 45° because root can grow over the transparent surface. Figure 5.1C shows the above and below ground plant biomass visible through rhizobox window to enable monitoring of growth and RSA; figure 5.1D shows processed root images showing full RSA detail. The plant shown is grown in 160ppm Cr(VI) 45 days after germination.

Figure 5.1 shows a detailed experimental setup.



5.1.A : Final product dimensions can vary ±0.5 mm (on all axes) for technical reasons.



5.1.B : Rhizo-box incline at 45°

5.3.C : Root images (2021-09-24) (45th day after germination) at 160 ppm treatment concentration

Figure 5.1: Rhizobox set-up.

5.2.4 Laboratory growth chamber

The experiment was conducted in 2021 in a growth chamber set at 27 ± 0.5 °C temperature and 12 h/12 h day/night photoperiod with 300 mmol m^{-2} s^{-1} photon flux intensity (1000W LED Grow Light, MIVARRS® 2x2 ft Plant Grow Lights Full Spectrum) at the plant level with humidity control (75 to 85 %). As the plant can grow in water logged condition, we alaways saturated the soil and as the pots were selled no leachet were drained from the pots.

5.2.5 Root system analysis (RSA)

Plant roots were photographed with a Canon 60D® (18-megapixel) camera with a 50 mm prime lens at 5-day intervals through the transparent window of the rhizobox. As the roots of *S. cannabina* present different colours and some sections resembled that of the soil (figure 5.1), it was necessary to remove the background image interference using image processing software Adobe Photoshop and Image-J program (Corzo Remigio et al., 2021)

5.2.6 Soil sample preparation and Cr(VI) analysis

After considerable plant growth (45 days), the plants were detached from the soil and soil samples were dried at 80°C for 48 hours to a constant weight. Alkaline digestion method (EPA method 3060A) (Alyazouri et al., 2014) was used to determine Cr(VI). The extracted Cr(VI) was reacted with 1,5-diphenylcarbazide (ACS Reagent, Sigma-Aldrich, Gillingham, UK) in the presence of sulphuric acid and analysed using a UV-visible spectrometer (model: JENWAY spectrophotometer 6505) at a wavelength of 540 nm (de Oliveira et al., 2016; De Oliveira et al., 2014; Alyazouri et al., 2014, 2020).

5.2.7 Dissolved Organic Carbon (DOC) in soil

At the end of the experiment (after 45 days), rhizobox soils were air-dried and passed through a 0.25-mm sieve. 5g of soil was transferred into 50 mL centrifuge tubes, and 30 mL of de-ionised water was added (soil :water ratio of 1:6) (Gao et al., 2017; Zhu et al., 2018). After shaking the tubes for 24 h at room temperature, the samples were centrifuged at a speed of 4000 r min^{-1} for 20 min and then filtered through 0.45-mm filters. The liquid suspension, i.e., the extracted DOM solution, was preserved in the dark at -20°C until further use. DOM concentration was represented as dissolved organic carbon (DOC), which was measured with a total organic carbon analyser (Shimadzu, TOC-L, Japan).

5.2.8 Evaluation of root growth and Cr(VI) tolerance

Root growth was measured using tap root length, area of root coverage (from the processed photograph taken for RSA) and final root dry weight. We measured the dry weight of the root by rinsing (several times) the harvested root (after 45 days) with de-ionised water, and samples were dried at 80 °C for at least 48 hours to a constant weight. Growth measurements were used for evaluating the weight-to-length ratio (WLR) at day 45, heavy metal HM tolerance index (TI), and root growth ratio (RGR) resistance indicators, which are defined as follows (Baker 1987):

$$\text{weight to length ratio, WLR (mg } cm^{-1}) = \frac{\text{root biomass (DW, mg)}}{\text{root length (mm)}}$$

$$\text{HM tolerance index, TI} = \frac{\text{root length with Cr(VI)}}{\text{root length without Cr(VI) (control)}}$$

$$\text{Root growth ratio, RGR } (\%) = \frac{\text{plant biomass with Cr(VI) } (DW, mg)}{\text{plant biomass without Cr(VI) (DW, mg)(control)}} \times 100$$

5.2.9 Statistical analysis

The experiment used a randomized design with 8 different doses of Cr(VI) and 3 replications per dose. Each rhizobox was an experimental unit. Statistical analysis including calculation of average values and standard deviation (S.D.) were calculated by the Microsoft office Excel 2013. The data collected from the different parameters were compared among different doses of Cr(VI) by analysis of variance (ANOVA). The data were processed using SPSS (2025), and a Tukey (LSD) significance difference test was used to compare the means when the differences between treatments were significant ($p \leq 0.05$).

5.3 Results and Discussion

We observed no root growth (by visual inspection) during the 360 ppm Cr(VI) treatment experiment. Roots could only grow in ≤ 160 ppm Cr(VI) treatment. However, within the 0-160 Cr(VI) range, root growth decreased with increased Cr(VI) treatment ($p < 0.05$) (figure 5.2). Figure 5.3 shows the root distribution system for the seven different concentrations at eight consecutive times (one randomly selected replicate). It is clear that root growth is severely hindered by over 80 ppm Cr(VI) (figures 5.2 and 5.3). It has been well documented that root growth decreases with the increase of Cr(VI) because metals ion primarily targets the root rather than arial part (Srivastava et al., 2021; Adiloğlu and Göker, 2020; Patra et al., 2020) . Cr(VI) have a high effect on the root length and growth rather than other HM , possibly due to root surface damage, causing leakage of cell content and collapse of root hairs and epidermal cells (Srivastava et al., 2021; Adiloğlu and Göker, 2020;

Patra et al., 2020). Interestingly, we observed a slight increase in root length for 5 ppm Cr(VI) concentration (figure 5.2), and this enhanced growth under low HM concentrations has been documented elsewhere. Dixit et al., (2002) observed a minor enhancement in the root length of *Pisum sativum* 2 ppm Cr(VI), but a decline in root length by 18% at 20.7 ppm Cr(VI).

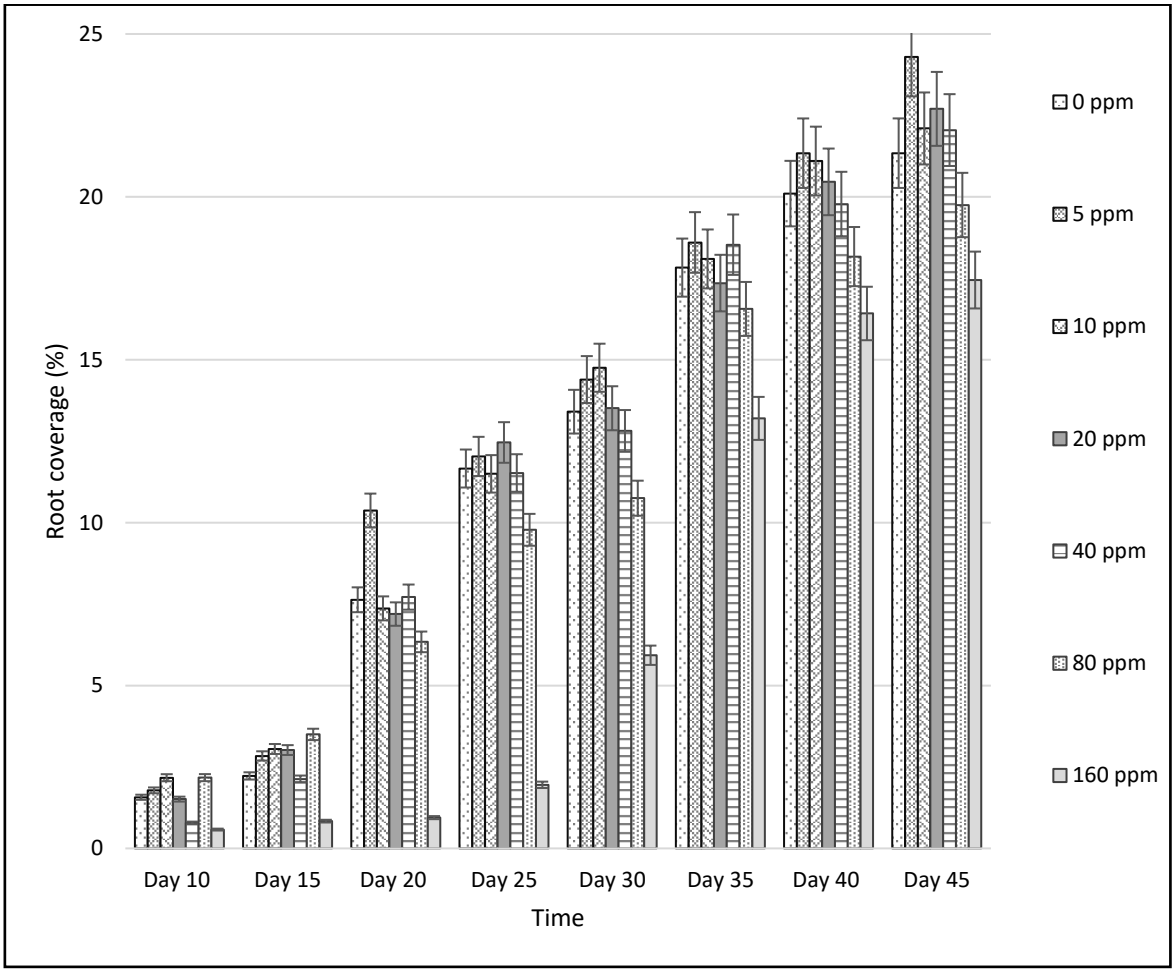


Figure 5.2: Average root area coverage (%) over time under increasing concentrations (ppm) of Cr(VI). Bars indicate standard error (n=3)

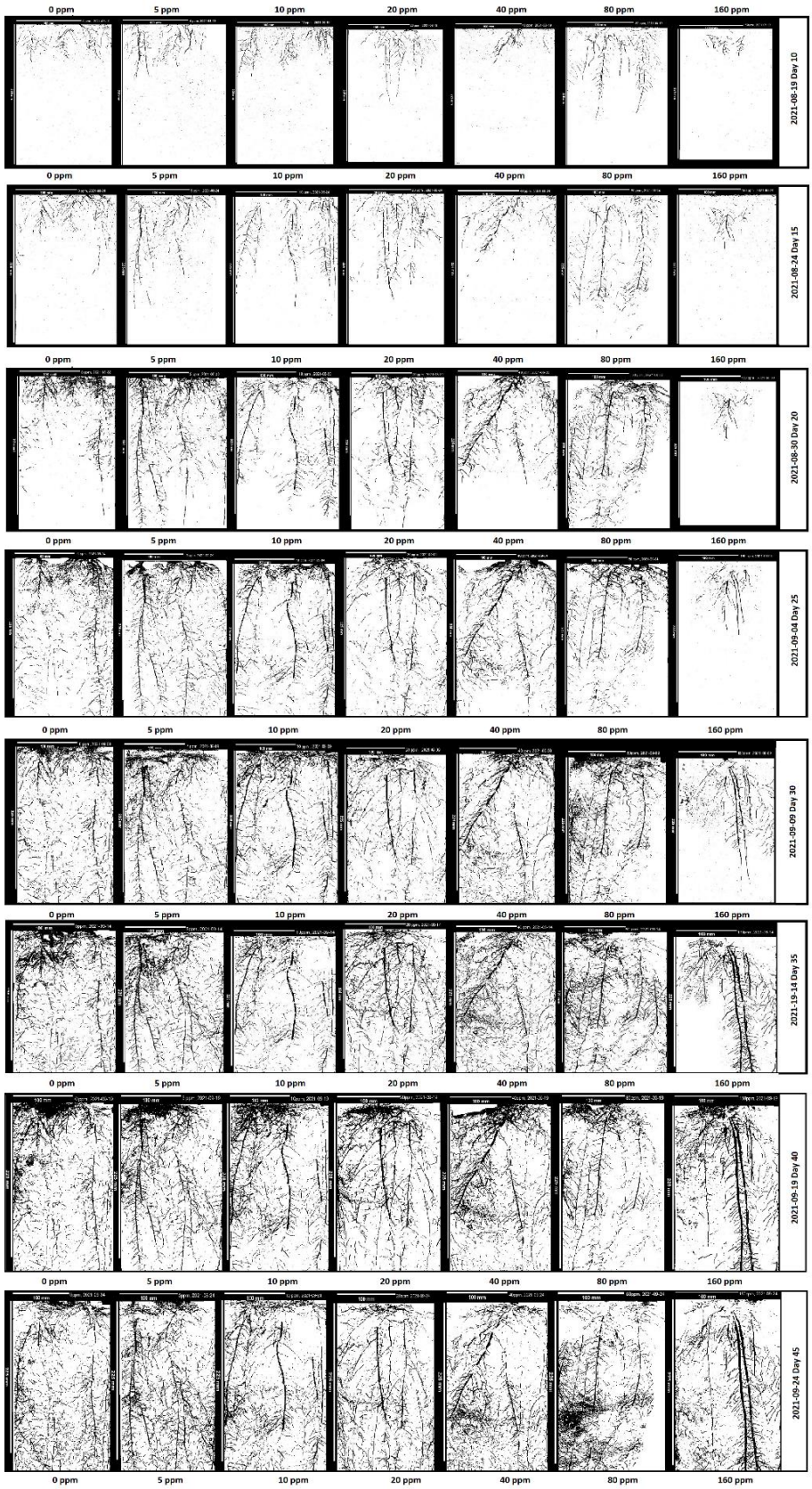


Figure 5.3: Root coverage over time (up to 210 mm) under increasing concentrations of Cr(VI).

As well as hindered root growth at 160 ppm Cr(VI), we also observed damage to the tip of the root after 20 days with subsequent regrowth and new root development after 25 days (figure 5.4); there was no visible root damage in treatments ≤ 80 ppm Cr(VI). Despite root tip damage (death) in the 160 ppm treatment, regrowth and new growth after 25 days suggests Cr(VI) had been effectively lowered, either by absorption by antecedent roots (since persished), and/or conversion (reduction) of Cr(VI) to less harmful Cr(III) by root exudates (Sinha et al., 2018; Sahoo et al., 2018; Wakeel and Xu, 2020).

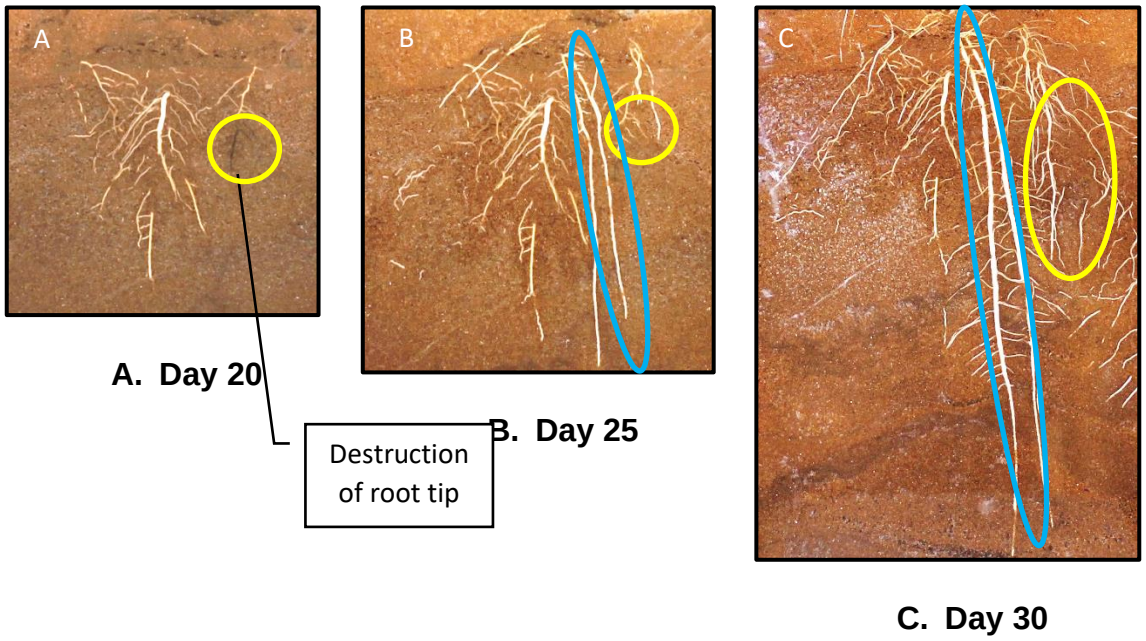


Figure 5.4: RSA images showing root damage and regrowth in 160 ppm Cr(VI) sample. A. Root death is visible, having occurred between day 15 and day 20. B. Root regrowth observed on day 25 (yellow circle) and new growth has established (blue oval). C. Root growth continued vigorously by day 30 .

Scanning electron microscope studies of roots affected by Cr show an increase in the growth of root hairs, and the relative proportion of pith and cortical tissue layers due to changes in speciation and mobility of Cr ions in the presence of DOC

(Suseela et al., 2002). During plant growth, organic acids are released, decreasing soil pH (Alyazouri et al., 2020); decomposition of organic compounds releases DOC, and plant roots release exudates, including oxidised chelating ligands (eg carboxylic acids), with potential to alter the form, availability and toxicity of chromium. Chromate efflux mechanisms have also been observed in some plants, which helps them survive Cr pollution in soil (Srivastava et al., 2021; Ranieri et al., 2020; Wakeel and Xu, 2020; Sinha et al., 2018). Overall, root growth decreased with an increase in Cr(VI) concentration (figure 5.5); and a strong negative correlation (r= - 0.98) was observed between DOC content (figure 5.6) and Cr(VI) (figures 5 and 6).

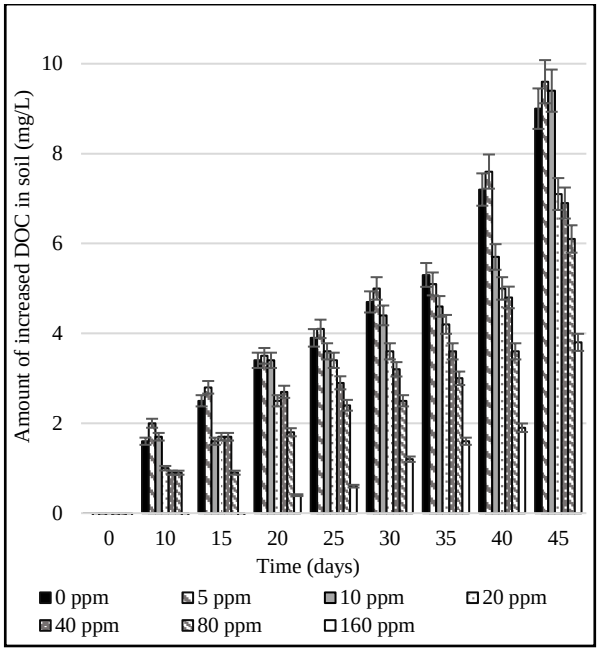


Figure 5.5: Changes in DOC during the experiment (initial concentration was 38.25 ± 0.15), n=3

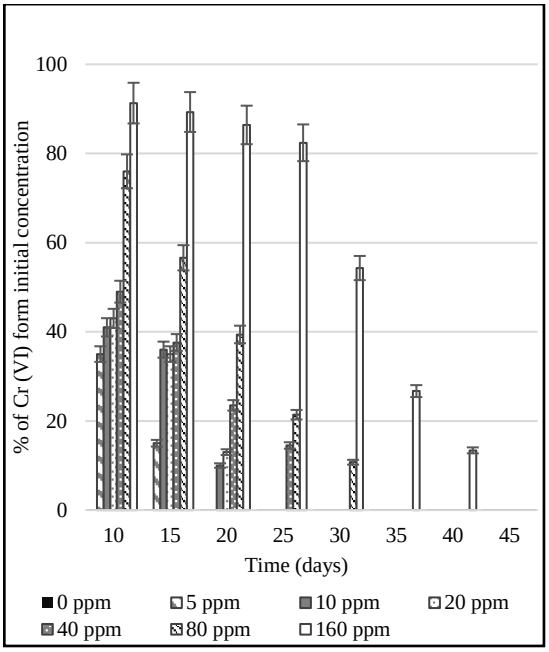


Figure 5.6: Soil-Cr(VI) during the experiment as a % of initial Cr(VI) dose, n=3.

Although a decrease of root length with increasing Cr(VI) dose in soil was observed, these differences were not significant in treatments up to 80 ppm, indicating the plants were tolerant up to 80 ppm Cr(VI) (figure 5.4).

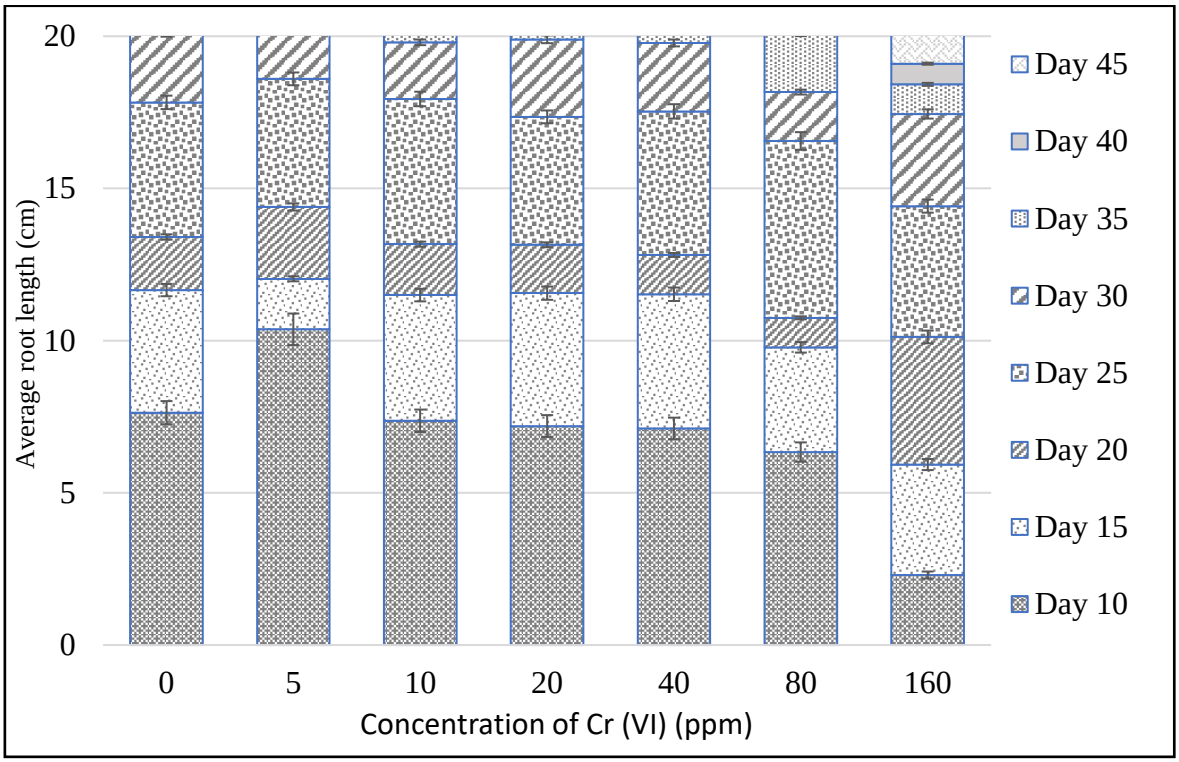


Figure 5.7: Average root length over time (up to 20 cm) under different concentrations (in ppm) of Cr(VI), n=9.

The weight-to-length ratio (WLR) and HM tolerance index (TI) are parameters used to characterize the effect of HM stress on root tissue as the first point of contact between plant and pollutant (Santiago-Cruz et al., 2014). The WLR for roots of plants grown in ≤ 80 ppm Cr(VI) was not significantly affected (85.7± 2 mg cm^{-1}) for plant roots grown in ≥ 160 ppm Cr(VI), WLR was significantly reduced (53.3 mg cm^{-1}), and this consequently diminished the production of plant biomass of roots. Researchers have identified that Cr(VI) can stop the cell growth in roots and thus decrease root growth and length; in addition to that, it also hinders nutrient transport and root hydration (Sahoo et al., 2018; Wakeel and Xu, 2020; Ertani et al., 2017).

These observations can be related to WLR of *S. cannabina*. Alternative HM resistance indicators demonstrate an increase in plant stress up to 80 ppm Cr(VI) treatment, with plant RGR and TI values significantly decreasing as Cr(VI) concentrations increased (figure 5.8),.

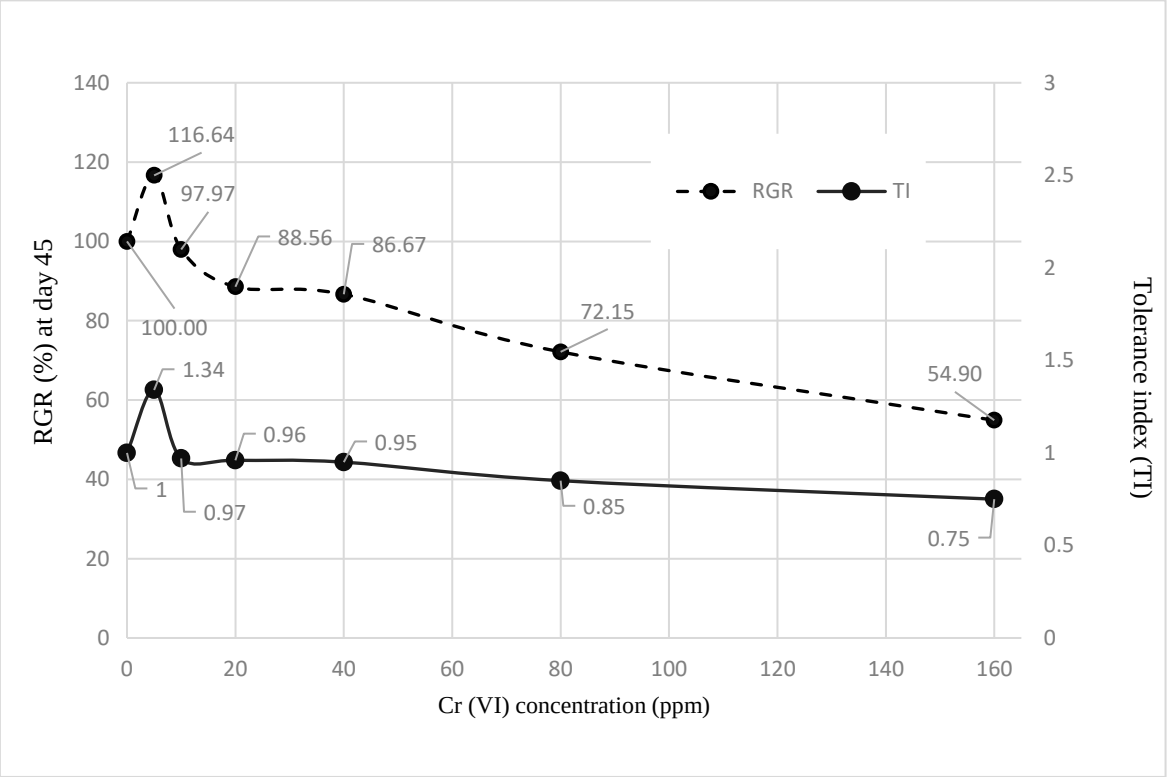


Figure 5.8: Root growth rate (RGR) and tolerance index (TI) after 45 days grown in different Cr(VI) in ppm dosed soil concentrations.

Figure 5.8 shows a U-shaped dose response curve for RGR during Cr(VI) exposure of *S. cannabina.* This shows growth stimulation at low concentrations (5 ppm Cr(VI)) and inhibition at higher doses (10-160 ppm Cr(VI)), in accordance with hormesis described by Poschenrieder et al. (2013). Although chromium is considered as a non-essential element for plant growth and development (Hayat et al., 2012; Wakeel and Xu, 2020), our study supports Dixit et al., (2002), where low concentration (≤ 2.5 ppm Cr(VI)) increases germination (and root growth) in *Pisum sativum* L. cv. Azad (Pea plant) compared to controls. Peralta et al., (2001) also

reported a stimulating effect on root elongation compared to the control for *Medicago sativa* L. (Alfalfa) at 5 ppm Cr(VI) treatment. Other work has also found low concentrations of heavy metals (cadmium, arsenic and copper) to have a minor stimulation effects on seed germination percentage (Lefèvre et al., 2009; Kjær et al., 1998; Chun-xi et al., 2007).

In this study, the decrease in root growth a more discrete while Cr(VI) dose increased from 80 to 160 ppm (Figure5.7). In hydroponic culture *Leersia hexandra* grown in 60 ppm (~1.15mM) Cr(III) showed no effect on the shoot and root biomass (Liu et al., 2011) , but *Typha angustifolia* while grown 1.0 mM Cr(VI) dosed soil recorded high decrease of dry weight tissue of shoot (30 %) and root (43 %) tissue after 30 days of culture (Bah et al., 2010).

5.4 Conclusion

S. cannabina root survived Cr(VI) dose as high as 160 ppm showed. Upto 80 ppm concentration both tap root and secondary root showed no damage but at 160 ppm less growth in secondary root was observed.

5.5 Reference

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Chapter 6 :

An assessment of the phytoremediation potential of *Sesbania cannabina* (Retz.) Poir. grown in hexavalent chromium contaminated soil.

Abstract

Hexavalent chromium is one of the most toxic heavy metals (HM) and it is harmful to living organisms but still its widely used in industrial activities. In this study, a legume plant, *Sesbania cannabina*, was considered for phytoremediation study of chromium (VI) contaminated soil. To assess the growth, tolerance, and phytoremediation ability of *S. cannabina,* a pot experiment was conducted under greenhouse conditions (simulated tropical conditions). The results showed that *S. cannabina* could tolerate and grow within concentrations of up to 175 ppm of Cr(VI) and was able to convert all Cr(VI) to the less toxic Cr(III). *S. cannabina* had bio-absorption coefficient (BAC) and translocation factor (TF) values <1 and root concentration factor (RCF) above 1. Thus, *S. cannabina* can be categorized exclusively as a phytosequestration species for Cr(VI). Pot experiments confirm that *S. cannabina* offers an alternate method for phytoremediation of Cr(VI) contaminated soil.

Keywords: *Sesbania cannabina*; phytoremediation; hexavalent chromium.

6.1 Introduction

The industrial revolution caused a substantial increase in the production of industrial waste and this, combined with globalisation, has resulted in the modern world facing serious environmental problems (Li et al., 2019; Yang et al., 2018). Production industries, agricultural and mining activities generate numerous toxic materials such as insecticides, organo-chloride pesticides, greases and oils, and heavy metals (HM), which are directly or indirectly released into the environment and contaminate the water and soil (Vardhan et al., 2019; Zwolak et al., 2019). HM severely threatens most living organisms, and contaminated agricultural soil disrupts crop yield and threatens human well-being (Stambulska et al., 2018; McComb et al., 2012a). Worldwide, researchers identified 5 million sites (500 million ha of land) as contaminated because the concentrations of HMs or metalloids are above permissible limits (Liu et al., 2018). In addition to health and environmental issues, HM contamination greatly impacts the global economy, and the estimated total loss in the global economy is more than 10 billion US dollar per annum (He et al., 2015). By definition, HMs are metals or metalloids with a density of over 5 g/cm^3 (Oves et al., 2012). Lead (Pb), chromium (Cr), mercury (Hg), cadmium (Cd), and arsenic (As) are HM known to be very noxious to human health even at a low dose (Yang et al., 2018; Zwolak et al., 2019). Other HMs (Fe, Mn, Cu, Zn, and Mo) are micronutrients, and the excessive amount of these metals in soil may not be harmful to humans or animals but can be very dangerous for plant growth and development (Zwolak et al., 2019).

Following the discovery of chromium from Crocoite ($PbCrO_4$, Siberian red ore) by Vauquelin in 1798, various industries use this chromium salt for different processes

(Shanker et al., 2005; Hayat et al., 2012b). These industries include metallurgy, rawhide tanning in leather processing, chrome plating, steel production, metal finishing, catalyst production (synthetic rubies), pigment manufacturing, and metal corrosion inhibitors (Hingston et al., 2001; Jeřábková et al., 2018; Kotas and Stasicka, 2000). The by-products cause extensive environmental pollution due to unregulated disposal in water bodies (Shanker et al., 2005; Hayat et al., 2012b). Naturally, chromium (as total chromium) can be abundant in soil with an average of 1 to 100 ppm, but its distribution is variable around the world (US :25 to 85 ppm; Japan: mean 87 ppm; Sweden: 74 ppm) (Zayed and Terry, 2003; Shanker et al., 2005). However, the permissible limit set by the different authorities for Cr(VI) in the soil is between 1 ppm to 21 ppm for residential, garden, or agricultural land and ≥ 41 ppm for commercial or industrial sites (CCME, 1999; DoEC, 2010; MEF, 2007; Tóth et al., 2016).

Chromium is a transition element with stable and unstable valence states (Cr(I) to Cr(VI)). Trivalent chromium Cr(III) and hexavalent chromium Cr(VI) are stable (Oliveira, 2012) but have different mobility, bioavailability, and toxicity. The solubility of Cr(VI) in water is very high and more toxic to living organisms than to Cr(III) (Hu et al., 2016; Kotas and Stasicka, 2000). The toxicity mechanism of chromium in plants is complex because plant uptake, translocation, and accumulation depend upon metal speciation and electrochemistry (Shanker et al., 2005; Hayat et al., 2012b). It has been reported for many plant species that chromium affects germination, root growth, and stem/leaf development (Zayed and Terry, 2003; Hayat et al., 2012b). Depletion in plant photosynthetic, antioxidant enzymes, nutrient imbalance, and oxidative stress in plants have also been recorded in plants

in response to increased concentrations of Cr in soil (Seneviratne et al., 2017; Samantaray et al., 1998).

Researchers worldwide have been looking for remediation options for this toxic Cr(VI) metal from the soil and have suggested several processes; among them, phytoremediation is considered less expensive and can be performed in situ (Peng and Guo, 2020; Srivastava et al., 2021). Many plants from different families have been studied, but very few species have been considered hyperaccumulators (Srivastava et al., 2021; Sinha et al., 2018). Zhang et al., (2007) introduced *Leersia hexandra* as a potential Cr hyper-accumulator. In their study, Cr(VI) reduction and sequestration were observed in hydroponic (batch) culture, and the highest bioaccumulation coefficients were found on the leaves: 72.1 after 45 days of treatment at 10 ppm Cr(VI). Chromium (total) accumulated in foliage was 597 ppm for Cr(VI) treatment (Zhang et al., 2007). However, while grown in ≥ 20 ppm of Cr(VI), a substantial decrease in foliage biomass has been observed (Zhang et al., 2007). *L. hexandra* swartz was therefore considered a potential plant for phyto-extraction, especially in large-area of (low-concentration) contaminated environments (Lin et al., 2018; Zhang et al., 2007).

Gardea-Torresdey et al., (2004) identified *Convolvulus arvensis* (Bindweed), a herbaceous perennial plant of the Convolvulaceae family, as a promising hyperaccumulator for Cr(VI). In this experiment, *C. arvensis was germinated* in different concentrations of Cr(VI) spiked (up to 80 ppm of Cr(VI)) agar-based nutrient mediums. The accumulated Cr in roots was about 20,000 ppm (DW) Cr(VI) and 2100 ppm (DW) Cr(VI) in foliage when the plant was allowed to grow in an agar-based nutrient medium spiked with 20 ppm Cr(VI) for two weeks. *Prosopis laevigata* (smooth mesquite) also showed a high accumulation of Cr(VI) while seeds were

germinated in tissue (batch) culture condition in modified Murashige–Skoog medium added with $K_2Cr_2O_7$ (0 - 353.6 ppm) at pH of 5.8 (Buendía-González et al., 2010). After 50 days, accumulation in roots reported 8090 ppm Cr(VI) (DW) and shoots contained 5461 ppm (DW) Cr(VI). According to the researcher, the translocation factor was recorded below 0.7 but due to high accumulation in roots the author suggested *P. laevigata* as a hyper-accumulator (Buendía-González et al., 2010). In all the studies, plants were grown in hydroponics or in an artificial substrate and low concentrations of Cr(VI). However, the search for plant species which able to germinate in Cr(VI) contaminated soil and have the potential to phytoremediate Cr(VI) contaminated soil at higher concentrations (> 20 ppm) is still ongoing.

Sesbania cannabina is a leguminous high-yield fodder crop (Sarwar et al., 2015) that has not been studied yet for Cr(VI) tolerance and uptake. It can grow in a wide range of climatic conditions (semi-arid to sub-humid), grows well even in marginal lands, tolerates a range of environmental conditions, including seasonally submerged soils, and is common in Bangladesh (Ren et al., 2019; Sarwar et al., 2015). The riverbank sediment of the Buriganga River (in Bangladesh) is contaminated with HMs (especially Cr) due to industrial activities (Nargis et al., 2018; Islam et al., 2015). Due to favourable climatic conditions, *S. cannabina* can grow naturally in these areas (Sarwar et al., 2015). Therefore, these attributes make it a viable candidate for phytoremediation of Cr(VI). The aims of this work were to (a) determine the effect of Cr(VI) on the growth and physiology of *S. cannabina* and b) assess the phytoremediation capacity of *S. cannabina* for Cr(VI) contaminated soil.

6.2 Materials and Methods

6.2.1 Soil preparation

Soil (sandy loam) was collected from a reputable supplier (Singletons Nurseries, Worcestershire, United Kingdom) meeting British standards for topsoil (BS 3882:2007). Characteristics of the soil are provided in table 6.1.

Table 6.1: Important parameters of soil used in this experiment (average values ± SD (n=3).

pH	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})	OM (%)	Sandy soil content (%)	Zn (ppm)	Cu (ppm)	Ni (ppm)	Cr (total) (ppm)	Cr(VI) (ppm)	Pb (ppm)
6.7 -7	62± 2	87± 3	412 ± 8	3.1 to 3.3	60 to 61%	110± 2.4	36± 1.2	20 ± 1.1	9± 1.2	< 0.1	5±0. 21

6.2.2 Experimental Procedure

A germinated seed of *Sesbania cannabina* was planted (1 cm depth) in each of 48 plastic pots (height 315 mm, volume 250 ml) containing 350 g (±0.5) uncontaminated soil. The bottom part of the pots was sealed to prevent contaminant loss by leaching because the solubility of Cr(VI) in water is very high (Hu et al., 2016; Kotas and Stasicka, 2000). There were six replicates for each treatment and one control (with no plants), including the control. The soil was dosed with Cr(VI) (as aqueous analytical grade (Merck) $K_2Cr_2O_7$) and mixed separately in each pot (before planting), giving a range of concentrations (0 (control), 25, 50, 75, 100, 125, 150, 175, and 200 ppm). The experiment was conducted under controlled conditions using vitopod® for 45 days in a 12/12h photoperiod (full spectrum light) and humidity of 75 to 85 % at 28°C constant temperature.

Figure 6.1: Plant growth after 15 days in a closed vitopod® growth chamber.

Plant growth after 45 days was recorded as shoot height, root length and biomass. At 45 days (after planting) plant (root, stem, and leaf) was harvested and prepared for Cr(VI) and total Cr (with ICP OES model: Agilent 7500ce) analysis along with proline and chlorophyll. Soil samples were also taken for Cr analysis.

6.2.3 Chlorophyll content

Fresh leaves were harvested 45 days after germination to determine photosynthetic pigments (Chlorophyll a, b, and total), according to Arnon, (1949). 40 mg of leaves were preserved in a sealed bottle (wrapped with dark paper) with 10ml (80%) acetone for 5 days in the refrigerator (at 4° C). After five days, the extract was centrifuged (5000 rpm for 10 min) and a spectrophotometer (model: JENWAY spectrophotometer 6505) was used to determine the optical density of the blank and the supernatant of the samples at wavelengths of 646 nm and 663 nm. According to Lichtenthaler and Wellburn, (1983), the following equations were used for the determination of chlorophyll content

$$C_a = 12.21\, A_{663} - 2.81 A_{646}$$

$$C_b = 20.13\, A_{646} - 5.03\, A_{663}$$

$$C_{total} = C_a + C_b$$

Where C_a is chlorophyll a, C_b is chlorophyll b, C_{total} is total chlorophyll in µg (ml of plant extract $^{-1}$), and A is the measured absorbance values at different wavelengths.

6.2.4 Proline content

Proline content was determined according to the protocol of Bates et al., (1973). Fresh leaves (500 mg) were crushed with 10 mL of 3% sulphosalicylic acid and centrifuged at 3000 rpm for 8 min. Two mL of supernatant were combined with 2 mL of acidic ninhydrin in a test tube. Acidic ninhydrin was prepared by 1.25 g ninhydrin (1,2,3-indantrione monohydrate), 30 mL glacial acetic acid, 20 mL of 6 M orthophosphoric acid, dissolved by vortexing and gentle warming and glacial acetic acid (99.9% acetic acid). The solution was incubated at 96° C for 60 minutes, and the reaction terminated in ice. After half an hour, we added 4 mL toluene to the test tube and shook vigorously. A spectrophotometer (model: JENWAY spectrophotometer 6505) was used to measure the optical density of the top red layer at 520 nm for proline content. In this experiment, we consider toluene a reference material.

6.2.5 Sample Preparation for Cr analysis

Plant and soil sample preparation for total Cr analysis

After 45 days from germination, the plants were harvested, and soil adhered to the roots was removed and preserved with pot soil using a deionized water (DI water) stream. The plants were split into leaves, stems, and roots, and all samples,

including soil, were dried (65°C for 72 hours) and grounded by a mortar and pestle (Alyazouri et al., 2020).

6.2.6 Analysis of chromium species

The following methods were used in chromium analysis:

(i) Total chromium: samples were acid digested [Acid digestion: oven-dried samples were crushed, and 0.1 g of powdered (homogenised) samples were placed into a digestion tube with 23 ml of HCl (con.) and 7 ml of HNO_3 (con.). After 12 hours, the digestion tube was placed in a heating block at 80° C for reflux for 2 hours and then filtered with a wetted filter (Whatman 41) paper into a 25 ml volumetric flask. An ionisation suppressant KCl (0.5 ml of 10%) is added to the filtered materials, and finally, the tube and filter paper are repeatedly washed with ultra-pure water and made volume up to the mark of a volumetric flask]; and the resulting solution analysed with ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy; model: Agilent 7500ce) (All chemicals were ICP grade).

(ii) Hexavalent chromium: samples were digested by alkaline digestion method [alkaline digestion: 2.5 ± 0.10 g of the sample were placed into a clean 250 mL digestion vessel and 50 mL ± 1 mL (dissolve 20.0 ± 0.05 g NaOH and 30.0 ± 0.05 g Na_2CO_3 in reagent water in a one-litre volumetric flask and diluted with DI water and maintained the pH 11.5 or greater,) of digestion solution were added to each digestion vessel. We also added approximately 400 mg of MgCl and 0.5 mL of 1.0 M phosphate buffer mixed for 5 min. After that, the digestion vessel containing the samples was heated to 90-95° C (for 60 min). After cooling, the contents (in beaker) were transferred quantitatively

to the filtration apparatus and filtered through a 0.45µm membrane filter. Slowly add 5.0 M nitric acid solution to the beaker dropwise and adjust the solution's pH to 7.5 ± 0.5. Transferred the vessel's contents quantitatively to a 100 mL volumetric flask and adjusted the sample volume to 100 mL (to the mark for the volumetric flask) with reagent water. Mix well. The sample digestates are now ready to be analysed] and the resulting solutions were analysed using a JENWAY spectrophotometer 6505 following EPA method 3060A and method 7196 (USEPA, 1996; Alyazouri et al., 2014; de Oliveira et al., 2016; De Oliveira et al., 2014).

(iii) Trivalent chromium (Cr(III)): we calculate Cr(III) by subtraction of Cr(VI) from total Cr (de Oliveira et al., 2016; De Oliveira et al., 2014; Alyazouri et al., 2014).

6.2.7 Phytoremediation potential

The phytoremediation potential of *S. cannabina* was calculated by using the following equation;

$$Bio - absorption\ Coefficient\ [BAC] = \frac{Metal\ concentration\ in\ shoot}{Metal\ concentration\ in\ soil}$$

$$Translocation\ Factor\ [TF] = \frac{Metal\ concentration\ in\ shoot}{Metal\ concentration\ in\ root}$$

$$Root\ Concentration\ Factor\ [RCF] = \frac{Metal\ concentration\ in\ root}{Metal\ concentration\ in\ soil}$$

6.2.8 Statistical tests

Chromium content in plant tissues (roots, stem, and leaves), were analysed and compared using one-way ANOVA and post-hoc Tukey's test ($p \leq 0.001$) to determine differences between treatments. Pearson's coefficient for correlation

was determined at a significance level of $p < 0.05$ to determine by using SPSS 25 software.

6.3 Results and Discussion

6.3.1 Status of Cr(VI) in soil and S. cannabina

S. cannabina was shown not to grow (died) at ≥ 200 ppm of Cr(VI); therefore, no accumulation data were obtained for that treatment. After 45 days, no Cr(VI) was detected in soil or in plant samples in all treatments (≤ 175 ppm). It was assumed that all Cr(VI) had been transformed to Cr(III) and Cr(VI) concentration in the pot containing Cr(VI) without plant and water remained unchanged. In all treatments (except ≥ 200 ppm), Chromium concentrations in the soil after harvesting showed a significant correlation ($P \leq 0.05$) with Cr concentration in *S. cannabina* root ($r = 0.910$), stem ($r = 0.732$), and leaf ($r = 0.789$) which means Cr accumulation increases with the increase of Cr(VI) concentration in soil (table 6.2).

Table 6.2: Mean ± SD (n=6) chromium (Cr, total) concentrations in *Sesbania cannabina* plant parts and soil (ppm). Values followed by the identical letter for each parameter show no significant difference from one another, as identified by Tukey's LSD ($p \leq 0.001$).

Dose	Root	Stem	Leaf	Soil
25 ppm	$22.05^{a} \pm 0.91$	$2.07^{a} \pm 0.08$	$0.50^{a} \pm 0.06$	$19.99^{a} \pm 0.55$
50 ppm	$29.97^{b} \pm 1.29$	$2.81^{b} \pm 0.10$	$0.83^{b} \pm 0.04$	$29.15^{b} \pm 0.73$
75 ppm	$38.30^{c} \pm 0.40$	$3.43^{c} \pm 0.14$	$1.15^{c} \pm 0.06$	$35.75^{c} \pm 0.32$
100 ppm	$64.22^{d} \pm 0.69$	$3.81^{d} \pm 0.09$	$1.28^{d} \pm 0.26$	$57.92^{d} \pm 0.76$
125 ppm	$96.37^{e} \pm 1.29$	$4.38^{e} \pm 0.16$	$1.46^{e} \pm 0.03$	$82.22^{e} \pm 0.66$
150 ppm	$145.29^{f} \pm 2.97$	$5.24^{f} \pm 0.09$	$1.60^{f} \pm 0.03$	$113.95^{f} \pm 1.91$
175 ppm	$163.68^{g} \pm 0.63$	$5.46^{g} \pm 0.17$	$1.84^{g} \pm 0.04$	$123.98^{g} \pm 0.55$

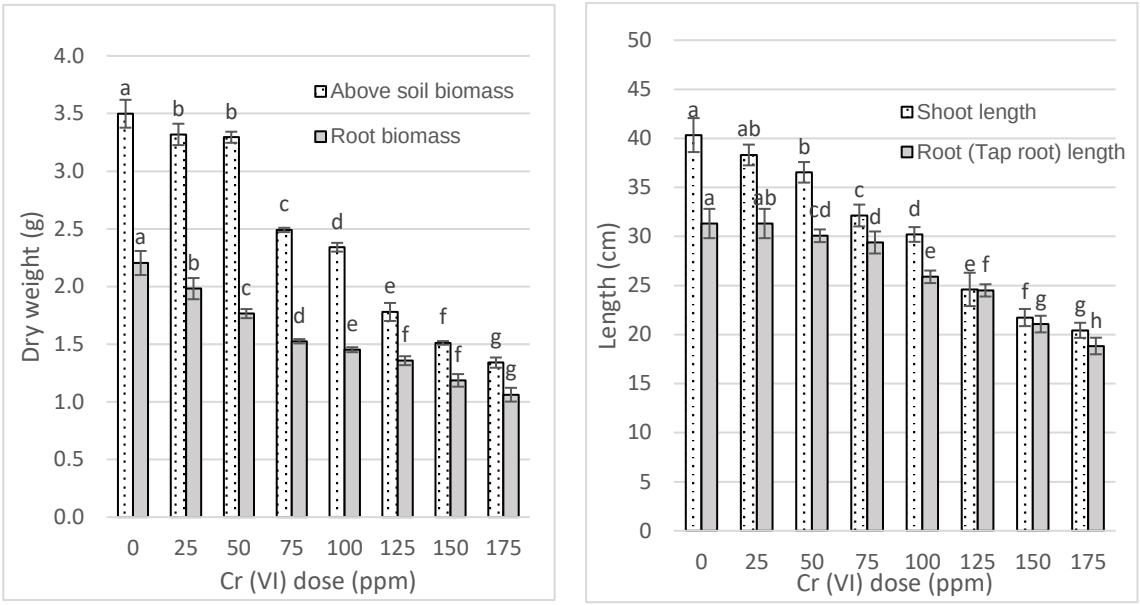
Maximum accumulation of Cr was detected (163.68±0.63 ppm) in the roots for 175 ppm treatment, but translocation of Cr from root to above surface biomass (stem and leaves) was negligible (9.4 to 3.2 %), and percentages of translocation (table 6.3) decreased with increased concentration of Cr in soil. Several studies of phytoremediation of Cr for different plant species show similar results; the root accumulates a major portion of total accumulated chromium and translocates a small portion to the stem (Liu et al., 2008; Vernay et al., 2007; Raimondi et al., 2020). Liu et al., (2008) showed that *Amaranthus viridis* L. (in hydroponic culture) accumulation of Cr was greater in the root (2624.39 ± 9.75 ppm) than in the stem (1626.04 ppm) for 10 ppm treatment concentration. Vernay et al., (2007) also recorded that *Lolium perenne* grown in Cr(VI) accumulated ten-fold higher Cr content in roots than leaves. *Prosopis laevigata* (smooth mesquite), a potential hyperaccumulator, also showed higher chromium accumulation in roots [8090 ppm Cr(VI) (DW)] than stems [(5461 ppm (DW) Cr(VI)] (Buendía-González et al., 2010). Studies on *Sesbania* spp. shows similar findings for different types of metal [e.g., *S. virgata* (for As, Cu, Zn, Cr) (Branzini et al., 2012), *S. grandiflora* (for Pb, Hg) (Malar et al., 2014), *S. exaltata* (for Pb) (McComb et al., 2012b)] where plant accumulated higher concentration of metal in roots compared to stems.

In this experiment, Cr(VI) accumulation in the different parts of the plant was root > stem > leaf, and root accumulated 10.6 to 31.27 times higher Cr than stem. Overall, Cr removal percentages from soil were observed as follows 25ppm (20±1.2 %), 50ppm (41.7±1.4%), 75ppm (52±2.1%), 100ppm (42.1±3%), 125ppm (34.2±3.2%), 150ppm (24±3%) and 175ppm (29.2±2.15%). Percentages of Cr removal slowly increase up to 75 ppm and then decreases with the increase in concentration.

Roots first come into contact with contaminants when grown in polluted soil (Shahid et al., 2017; Ertani et al., 2017), and root vacuoles play a vital role among the plant's several Cr(VI) uptake mechanisms. It has been shown that the root vacuole is the plant's largest organelle, and it has various significant and diverse functions, including cellular waste degradation and the storage of ions (Kaiser and Scheuring, 2020). Shanker et al., (2005) state that as a part of the natural toxicity response of plants, accumulated Cr is immobilized in the root cell vacuoles and converts the Cr(VI) into a less toxic form. Therefore, a high level of Cr in the root is probably linked to detoxification (or stabilization) of the Cr in the roots and also prevents translocation of Cr to the more sensitive parts of the plant (e.g., stem and leaf).

6.3.2 Cr(VI) effect on S. cannabina growth

Plant growth and biomass are key parameters for evaluating tolerance and adaptation to external stress (Shanker et al., 2005; Ertani et al., 2017). In this study, added Cr(VI) inhibited biomass production and growth of *S. cannabina* (figure 6.2) with an increased stress response with an increase in Cr(VI) concentration. No significant reduction of plant biomass (above the soil biomass) was observed up to 50 ppm Cr(VI) (dose), but ≥ 75 ppm plant biomass decreases with the increase of Cr(VI) concentration in the soil (figure 6.2. A). On the other hand, dry root biomass shows a more consistent decrease with increased Cr(VI) concentration in soil. In addition, less significant changes were observed in plant length (shoot length) and root length (tap root) up to 50 ppm Cr(VI), and in both cases, length reduction was observed with an increase of concentration ≥ 75 ppm of Cr(VI) in soil.



6.2. A **6.2. B**

Figure 6.2: 6.**2.A.** Above ground (leaves and stems) and root biomass (dry weight in g) for each Cr(VI) treatment in *S. cannabina* after 45 d of growth and 6.**2.B.** Plant length (shoot length) and root length (tap root) for each Cr(VI) treatment in *S. cannabina* after 45 d of growth. Error bars are standard error (n =6). Identical letters in the same colour bar show no significant difference, as detected by Tukey's LSD ($p \leq 0.001$).

In a similar study, Saravanan et al., (2019) observed the phytoremediation capability of *Vigna mungo* (black gram) at various concentrations of Cr(VI) (5, 25, 50, 75, 100 ppm) in soil and observed a decrease in root length (from 22.01±1.56 cm at five ppm to 1.46±0.11 cm at 100ppm) and shoot length (from 39.05±0.85 cm at 5 ppm to 12.36±0.88 cm at 100ppm). Ramana et al., (2017) studied *Agave americana* (Century Plant) for Cr(VI) phytoremediation (0 to 200 ppm) and observed a reduction of root biomass (from 3.75 g plant $^{-1}$ to 0.89 g plant $^{-1}$) and leaves (from 16.88 g plant $^{-1}$ to 2.27 g plant $^{-1}$). Cr(VI) does not play any role in plant growth at high concentrations (varies from species to species), and inhibits plant growth (Shanker et al., 2005). A study conducted by Vajpayee et al., (2001) to assess the

toxicity and chromium accumulation of Cr(VI) on *Vallisneria spiralis* reported biomass (dry) production in the nutrient medium was heavily hindered by Cr(VI) ≥ 2.5 ppm. Hydroponically cultivated cabbage plants grown in ≥ 10 ppm Cr(VI) shows a decrease in the whole plant's dry weight from 88.4 g/plant in control to 28.4 g/plant (Hara and Sonoda, 1979).

In this experiment, we observed no effect in plant growth ≤ 50 ppm of Cr(VI) (figure 6.2); however, ≥75 ppm showed a gradual decrease in biomass, root, and plant growth with an increasing Cr(VI) in soil and unable to grow ≥ 200 ppm Cr(VI). The highest dry weight value was recorded for root and above soil plant biomass among treatments at ≤50 ppm concentrations (figure 6.2) of Cr(VI). There are no significant changes in tap root length at ≤ 50 ppm (figure 6.2. B), but significant changes were observed in root biomass (2. A) at ≤ 50 ppm concentrations according to Tukey's LSD ($p \leq 0.001$) because less growth observed in lateral root ≥ 25 ppm compared to control.

6.3.3 Cr(VI) effect on the photosynthetic pigment of S. cannabina

Plant enzyme functions are disrupted when grown in HM (above toxic levels) contaminated soil (Varun et al., 2017; Srivastava et al., 2021; Sinha et al., 2018). In particular, toxic HMs affect photosynthetic processes, which negatively affect plant development and productivity (Shahid et al., 2017; Srivastava et al., 2021). It is also well-established that chromium toxicity in plants decreases the chlorophyll-a, chlorophyll-b, and total chlorophyll content (Shanker et al., 2005; Panda and Choudhury, 2005)

In our study, results clearly showed that photosynthetic pigments in plant leaves declined with an increasing concentration of Cr(VI) in soil (figure 6.3), and a

significant decline ($p < 0.05$) of chlorophyll-a and total chlorophyll concentration was observed at ≥ 75 ppm dose. The chlorophyll-a in *S. cannabina* leaves was significantly ($p < 0.05$) decreased from 0 ppm to a higher dose, but between 50 ppm and 75 ppm changes were insignificant. The chlorophyll-b in *S. cannabina* leaves reduced with an increase in Cr(VI) dose, but no significant difference was observed ($p < 0.05$) ≥ 100 ppm dose. The total chlorophyll content significantly ($p < 0.05$) decreases from 0 ppm dose to a higher dose (figure 6.3).

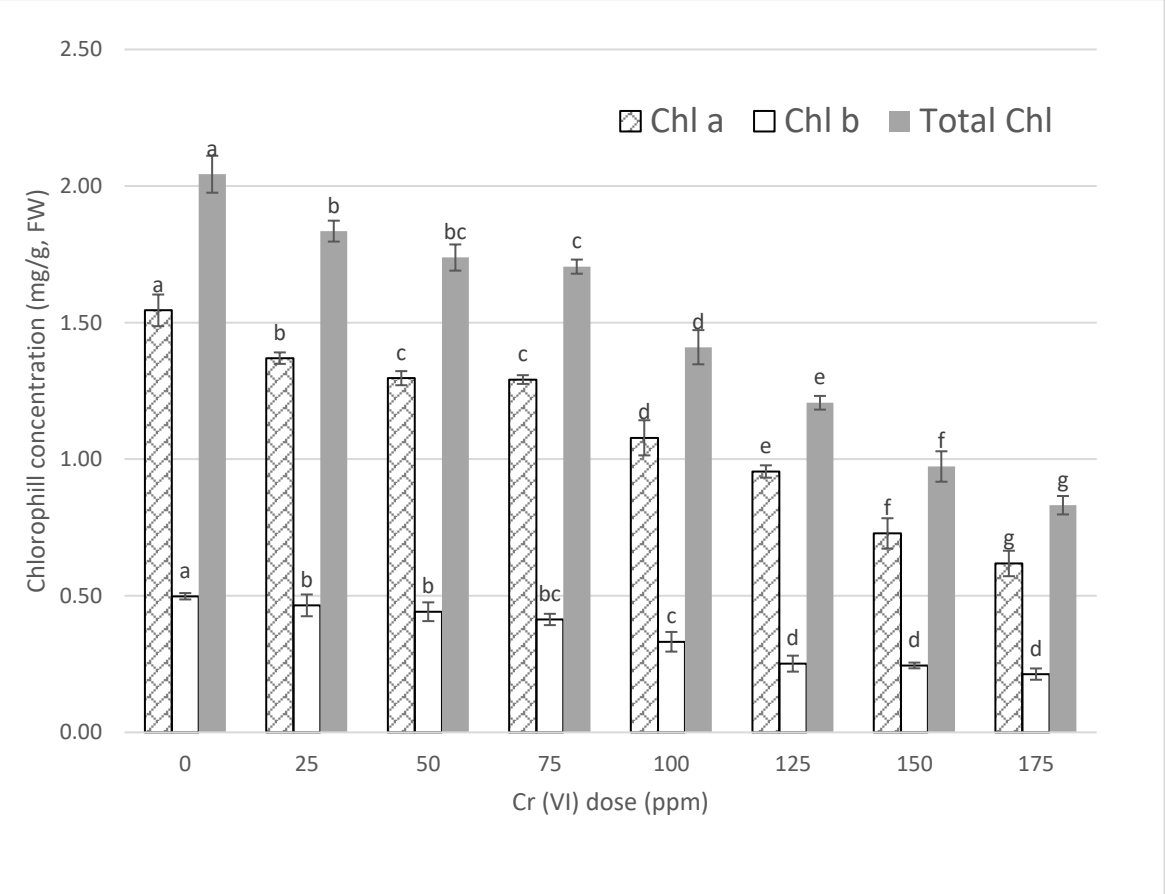


Figure 6.3: Response of different Cr(VI) concentrations on photosynthetic pigments (chlorophyll-a, chlorophyll-b, and total chlorophyll) in *S. cannabina* after 45 d of growth. Error bars are standard error (n =6). Identical letters in the same column/color bar show no significant difference, as detected by Tukey's LSD ($p \leq 0.001$).

Plant photosynthesis is disrupted or even stopped while grown in chromium (VI) contaminated soil due to ultrastructural changes in the chloroplast (Shahid et al., 2017). Many plant species also exhibit similar disruption in the photosynthetic process while grown in Cr(VI) contaminated soil, such as *Ocimum tenuiflorum* (Rai et al., 2004), *Hibiscus esculentus* (Amin et al., 2013) and *Lemna minor* (Uysal, 2013) Studies on several crop plants have shown chromium disruption of photosynthesis activities by inhibiting electron transport, disruption of Calvin cycle enzyme activation and decreasing CO_2 fixation (Panda and Choudhury, 2005; Rocchetta and Küpper, 2009). In this experiment, Cr(VI) significantly reduced total chlorophyll with increased Cr(VI) dose in soil.

6.3.4 Cr(VI) effect on proline content of S. cannabina

Proline is a proteinogenic amino acid which plays vital functions in leaves to regulate water loss, scavenge free radicals, protect enzymes and sub-cellular structures (Trovato et al., 2008; Liang et al., 2013; Kishor et al., 2015). Research has identified positive correlations between proline accumulation and plant stress (Hosseinifard et al., 2022; Aslam, 2017). According to Kishor et al., (2015), HM stress in plants elevated the proline content in the plant. At HM stress conditions in the plant, proline protects the plant by metal chelation and antioxidative defence (Hayat et al., 2012a; Pinho and Ladeiro, 2012).

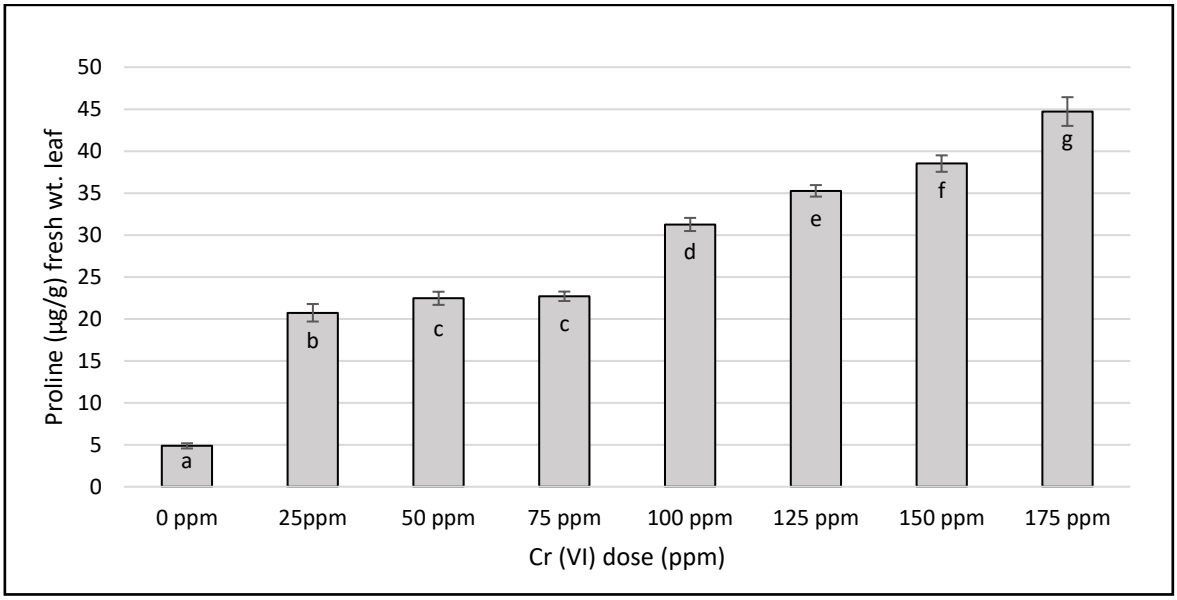


Figure 6.4: Effect of Cr concentration on proline content (µg/g) in *S. cannabina* after 45 d of growth. Error bars are standard errors (n =6). values followed by the identical letter for each dose are not significantly different from one another, as identified by Tukey's LSD ($p \leq 0.001$)

In this study, *S. cannabina* in all doses showed elevated proline accumulation than the control (figure 6.4), and proline level increased with increased Cr(VI). At 175 ppm dose, we recorded approximately 5 times higher than the control for 25 ppm to 75 ppm and at 175 ppm dose, about 9 times higher proline than the control (0 ppm dose). In a similar study, an increase in proline accumulation in tissues of *Ocimum tenuiflorum* L. exposed to Cr(VI) has also been observed, and the highest proline (600 µmole g^{-1}) was detected at 29.2 ppm Cr(VI) at a concentration (29.2 ppm) (Rai et al.,2004). Various research concludes proline accumulation increases in higher and lower plants for metal stress (Hayat et al., 2012a) but differs from species to species in response to different metals.

6.3.5 Chromium phytoremediation potential of S. cannabina

In phytoremediation research, bio-absorption coefficient [BAC], translocation factor [TF], and root concentration factor [RCF] were calculated to understand the plant's potential for phyto-management of HM contamination (Razmi et al., 2021; Mahmood-ul-Hassan et al., 2017).

Table 6.3: BAC, TF, and RCF of *S. cannabina* for Cr(VI) phytoremediation. Each value is the mean of six replicates (n = 6); values followed by the identical letter for each parameter are not significantly different from one another, as identified by Tukey's LSD ($p \leq 0.001$)

Dose	Bio-absorption coefficient [BAC]	Translocation factor [TF]	Root concentration factor [RCF]
25 ppm	0.1038$^{\mathbf{a}}$±0.0047	0.0941^{a}±0.0033	1.1036^{a}±0.0523
50 ppm	0.0963$^{\mathbf{b}}$±0.0029	0.0938^{b}±0.0045	1.0286^{b}±0.0464
75 ppm	0.0961$^{\mathbf{b}}$±0.0041	0.0897^{c}±0.0040	1.0715^{c}±0.0183
100 ppm	0.0658$^{\mathbf{c}}$±0.0018	0.0593^{d}±0.0018	1.1088^{a}±0.0105
125 ppm	0.0533$^{\mathbf{d}}$±0.0019	0.0455^{e}±0.0019	1.1720^{d}±0.0139
150 ppm	0.0459$^{\mathbf{e}}$±0.0008	0.0360^{f}±0.0009	1.2753^{e}±0.0290
175 ppm	0.0440$^{\mathbf{f}}$±0.0012	0.0334^{g}±0.0011	1.3202^{f}±0.0093

Plants with BAC and TF values >1 are considered potential phytoextractors, whereas those with RCF > 1 and TF < 1 are considered promising phyto-stabiliser (Saravanan et al., 2019; Gautam et al., 2017). In this experiment, we observed, *S. cannabina* can grow or tolerate ≤ 175 ppm Cr(VI) concentrations which indicates the capacity of the plants to grow in Cr(VI) contaminated soil. Despite substantial chromium accumulation in the root, a reduction in biomass (figure 6.2), a decrease in chlorophyll content (figure 6.3), and an increase in proline (figure 6.4) indicate that the plant is able to survive Cr(VI) ions up to 175 ppm. We also observed that

studied plants accumulate higher concentrations of Cr in roots compared to shoots (table 6.2), even when the soil contains a low concentration of Cr(VI).

S. cannabina had BAC and TF values all <1, and RCF is above 1 (table 6.3). *S. cannabina* can be categorized exclusively as a phyto-stabiliser for Cr(VI). In addition to that, the most promising outcome of this experiment is *S. cannabina* (root) can convert all Cr(VI) into Cr(III) at 45 days but is unable to grow ≥ 200 ppm Cr (V). According to USEPA, (2000) recommendations, a suitable species for phytoremediation must follow one of the conditions (1) a low yield with higher HM accumulation capacity or (2) a high yield with moderate HM uptake capacity. In this experiment, we observed chromium accumulation in plant tissue, and the ability to convert Cr(VI) to less toxic Cr(III), which makes this plant a suitable candidate for Cr(VI) phytoremediation.

These results suggest that *S. cannabina* can be used in phytoremediation of Cr(VI) contaminated soil (up to 175 ppm).

6.4 Conclusion

In this study, we observed *Sesbania cannabina* could reach up to (a height) 40±2 cm within 45 days. Based on its capacity for the reduction of Cr(VI) to Cr(III) and tolerance for Cr(VI), this studied plant can be considered a suitable phytoremediation tool for Cr(VI) contaminated soils in a short time with consecutive flushes.

6.5 References

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Chapter 7:

Conclusion & Recommendations

7.1 Conclusions

From this study, it can be concluded that:

- Chapter 2: Among the sampling points, riverbank sediment of the Dhaleshwari River near the tannery site demonstrated the highest pollution with chromium (VI) (in 2019 and 2021) and the classified as a carcinogen by the World Health Organisation and above the permissible limit (table 1.1). This pollution level calls for immediate action for remediation, and an in situ method such as phytoremediation can be a viable option to remove this Cr(VI) from the river bank sediment.
- Chapter 3: Achieving maximum germination before the photo-toxicity study of Cr(VI) is crucial. In summary, the results of this study section depict that *S. cannabina* can germinate and emerge under a wide range of conditions. But maximum germination (98±1.23%) is achieved while treating with 6% (v/v) H_2O_2 (5min) and 65°C hot water (5min); seeds of *S. cannabina* germinate rapidly between 25°C to 30°C. The emergence of *S. cannabina* from deeper burial in the soil (up to 8 cm) indicates that these seeds did not require any special technique for cultivation.
- Chapter 4: After achieving the condition for maximum germination, in this study section, we try to assess the Cr(VI) concentration in which the seeds of *S. cannabina* can germinate and grow. The results provide information about the plant's ability for Cr(VI) phytoremediation of contaminated land or sediment. Cr(VI) dose up to 100 ppm *S. cannabina* seeds can germinate without affecting root radicle growth. On the other hand, germination percentages and root elongation were hindered by ≥ 250 ppm. Again, according to germination

percentage results, *S. cannabina* seeds can germinate even up to 500 ppm of Cr(VI) concentration, but considering root elongation study and confocal image, it was clear that germination and root growth stopped ≥ 250 ppm after 96 h. Finally, it can be concluded that *S. cannabina* can germinate and grow in a medium containing ≤ 175 ppm concentration of Cr(VI).

- Chapter 5: As from the previous section, Cr(VI) directly affects the root radicle. In this study section, we observed that the root system of *S. cannabina* behaves under 0 to 360 ppm concentrations of Cr(VI) using rhizobox. At 360 ppm concentration, germinated seeds of *S. cannabina* could not grow. However, under 0-80 ppm concentrations, no significant change was observed in the root growth (length). At 160 ppm, root growth was reduced by about 55±0.65% at 25 days and 35±0.25 % at 45 days compared to plants grown at 0 ppm. After 45 days, no chromium (VI) was detected in the soil for (0 to 160 ppm) in comparison with the control (with no plants), where no changes in Cr(VI) were observed. The absence of Cr(VI) in the soil after 45 days suggests that *S. cannabina* can be a candidate for phytoremediation of soils containing up to 160 ppm Cr(VI).
- Chapter 6: After studying the germination and root growth under Cr(VI), we finally conducted a phytoremediation study (0 to 200 ppm, Cr(VI)). During the investigation, no effect on plant physiological changes was observed up to 100 Cr(VI) treatment. The results showed that *S. cannabina* could tolerate and grow within concentrations of up to 175 ppm of Cr(VI) and was able to convert all Cr(VI) to the less toxic Cr(III) (discussed in chapter 6). *S. cannabina* had bio-absorption coefficient (BAC) and translocation factor (TF) values <1 and root concentration factor (RCF) above 1. Thus, *S. cannabina* can be

categorised exclusively as a phytosequestration species for Cr(VI). Pot experiments confirm that *S. cannabina* offers an alternate method for phytoremediation of Cr(VI) contaminated soil. *Sesbania cannabina* could grow height of over 40±2 cm within 45 days. Based on its dense growth, reduction of Cr(VI) to Cr(III), ample biomass, tolerance, and being this legume crop can be used in phytoremediation Cr(VI) contaminated soil.

- Finally, S. cannabina can be used for remediation of Cr(VI) (≤ 175 ppm) contaminated site.

7.2 Limitations of this study

- As Cr(VI) is highly mobile, there might be a chance that Cr(VI) can be drained in the bottom of the sealed pot and thus have less effect on the initial seedling growth.
- In this experiment, we did not consider the other contaminants (interference) available in the industrial contaminated site because we used non-toxic soil.
- We also did not observe the effect of rhizobium bacteria on Cr(VI) remediation; however, we did not observe any nodule growth up to 45 days.

7.3 Recommendations

- Chromium (VI) in the riverbank sediment of the Dhaleshwari River of Bangladesh contaminated with Cr(VI) by tannery industries poses a severe threat to the local environment, including residential and agricultural areas, and must be sustainably managed urgently.

- Phytosequestration appears to be a promising, low-cost solution and can easily be applied in the remediation of Cr(VI) in the riverbank sediment of the Dhaleshwari River of Bangladesh. However, we recommend further research for additional contaminants from tannery industries such as organics.
- Leguminous plants like *S. cannabina* may be appropriate for other regions since they have the potential to tolerate the high as Cr(VI) (≤ 175 ppm) contaminated in soil, but this requires further investigation.
- Environmental regulations should be developed (especially for riverbank sediment pollution) and enforced because many industries do not implement these regulations in developing countries like Bangladesh.
- This study suggests the need to conduct further investigations on the effect of sulfate on Cr(VI) phytoremediation and the impact of counter-cation on the uptake on associated contaminant anions such as chromate.
- After phytoremediation, plant material could be used in anaerobic digestion for CH_4 production. By-product slurry can be used as bio-fertiliser because it will contain Cr(III), which is less toxic even in high concentrations. In addition to that, by using bioleaching methods, it is possible to recover all chromium.