CHROMIUM (VI) UPTAKE AND TOLERANCE POTENTIAL IN *AMARANTHUS DUBIUS* MART. EX THELL.: EFFECT ON GENOTOXICITY, CELL STRUCTURE, ANTIOXIDATIVE ACTIVITY, MINERAL NUTRITION, GROWTH AND NUTRITIONAL VALUE

Thesis submitted to the University of Calicut

in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN BOTANY

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This is to certify that the thesis entitled, **"Chromium (VI) Uptake and Tolerance Potential in *Amaranthus dubius* Mart. ex Thell.: Effect on Genotoxicity, Cell structure, Antioxidative activity, Mineral Nutrition, Growth and Nutritional Value"** submitted to the University of Calicut, for the award of the degree of **Doctor of Philosophy** in **Botany**, is a bona fide record of the original research work carried out by **Ms. SIMI R.,** at the PG Department of Botany & Research, Sree Narayana College, Nattika, Thrissur, Kerala, affiliated to the University of Calicut, Kerala under my supervision and guidance and no part of the present work has formed the basis for the award of any other degree/diploma to any candidate of any University previously.

Dr. Subin. M. P
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Forwarded through

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26/12/2022

The Principal & Head of the Research

DECLARATION

I, Simi R., hereby declare that the thesis entitled "**Chromium (VI) Uptake and Tolerance Potential in *Amaranthus dubius* Mart. ex Thell.: Effect on Genotoxicity, Cell structure, Antioxidative activity, Mineral Nutrition, Growth and Nutritional Value"** submitted to the **University of Calicut**, for the award of the degree of **Doctor of Philosophy** in **Botany** is a bonafide record of the original research work carried out by me under the supervision and guidance of Dr.Subin M. P., Associate Professor, PG Department of Botany & Research, Sree Narayana College, Nattika, Thrissur and that it has not been submitted earlier either in part or full for the award of any degree/diploma to any candidate of any University.

Sree Narayana College, Nattika **SIMI R.**

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CHAPTER-1

GENERAL INTRODUCTION, RESEARCH PROBLEM AND OBJECTIVE OF THE STUDY

GENERAL INTRODUCTION

The interactions of crop plant with the environment on the basis of physiochemical and molecular parameters has been an area of remarkable interest in the recent years. It is identified that, among the abiotic stresses which have hazardous impacts in plants, heavy metal toxicity plays a crucial role. The heavy metals are a group of nonbiodegradable, persistent inorganic chemical constituents having atomic mass over 20 and the density higher than 4-5g/cm^3 which cause cytotoxic, genotoxic and mutagenic effects on plants, animals and humans (Hawkes, 1997; Saxena & Shekhawat, 2013). The two varieties of metals identified in the soil which referred to as essential micronutrients for normal plant growth (Fe, Mn, Zn, Cu, Mg, Mo, and Ni) and nonessential elements with unidentified biological and physiological activity (Cd, Sb, Cr, Pb, As, Co, Ag, Se, and Hg) (Rascio & Navari, 2011; Schutzend¨ubel & Polle, 2002; Zhou *et al*., 2014). However, existence of such non-essential elements in surplus can cause toxicity leading to reduction and inhibition of growth in plants because of various reasons (Zengin & Munzuroglu, 2005).

When available in higher concentrations above the admissible level, the toxic impact of heavy metals (HM) on plants is highly significant. There are several reports that the presence of many heavy metals at higher levels obstruct the vegetative growth and reduce the productiveness and quality of plants (Liu *et al*., 2003). Toxic levels of HMs hinder the normal functioning of the plants and obstruct the metabolic processes in many ways such as disturbing and displacing building blocks of protein structure as a result of the development of bonds between HMs and sulfhydryl groups [Hall, 2002], inhibiting functional groups of main cellular molecules (Hossain *et al*., 2012), displacing or interrupting the efficacy of essential metals in biomolecules like pigments or enzymes (Ali *et al*., 2013), and unfavourably affecting the integrity of cytoplasmic membrane (Farid *et al*., 2013), causing a suppression of the important functions in plants like

photosynthesis, respiration and enzymatic exercises (Hossain *et al*., 2012). Enhanced production of reactive oxygen species (ROS) like superoxide free radicals, hydroxyl free radicals or non-free radical species (molecular forms) such as singlet oxygen and hydrogen peroxide (H_2O_2) and cytotoxic compounds like methylglyoxal (MG) that can create oxidative stress by upsetting the equilibrium between prooxidant and antioxidant homeostasis within the plant cells are all attributed to higher level of HMs (Zengin, & Munzuroglu, 2005; Hossain *et al*., 2012; Sytar *et al*., 2013). This situation implies the cause for a number of degenerative disorders like protein and lipid oxidation, leakage of ion, oxidative DNA attack, redox imbalance and adulteration of cell structure and membrane leading to the triggering of programmed cell death (PCD) pathways (Nagajyoti *et al*., 2013; Flora *et al*., 2008; Hatataand & Abdel, 2008; Rascio & Navari-Izzo, 2011).

The accelerated pace of industrialization along with irrational and unscientific discharge of industrial effluence is a major environmental hazard which leads to heavy metal pollution. The status of developing countries in this aspect is alarming as wastewater from industries containing untreated heavy metal is widely used for irrigation and also carelessly disposed of into open environments (UNIDO, 2002). The so discharged metals get deposited in soil profiles causing metal contamination in the long run. On getting absorbed by plants, these metals become toxic causing degradation in quality and reduction in growth and yield (Arun *et al*., 2005). Certain heavy metals like Zn, Fe, Cu, Mn are vital for proper plant growth and essential components of several enzymes with metabolic significance. On the other hand, metals such as Cr, Pb, Cd, As, Se and Al are toxic above specific threshold levels and are biologically insignificant.

Chromium is a chemical element with atomic number 24. Its molecular weight is 51.1 a.m.u. and density is 7.19 g/cm3 (Nriagu *et al*., 1988). The toxicity due to chromium is understood to hinder plant growth by developing ultrastructural modifications of the cell membrane and chloroplast, inducing chlorosis in the leaves, impairing root cells, decreasing pigment content, disrupting water relations and mineral nutrition, influencing photosynthesis, transpiration and assimilation of nitrogen and also by amending various

enzymatic activities (Shanker *et al*., 2009; Reale, 2016; Ali *et al*., 2015; Anjum *et al*., 2017). The redox balance in plants get disrupted as a result of excess production of reactive oxygen species (ROS) causing all the toxic effects of Cr (Anjum *et al*., 2017). Depending on the metal speciation which is accountable for the subsequent ingestion and ensuing toxicity in the plant structure, the impact of chromium contamination causes variation in the physiology, metabolism and growth of plant (Shanker *et al*., 2005). In contrary to other toxic contaminants, Cr has many oxidation states and of these, the trivalent Cr^{III} and the hexavalent Cr^{VI} are the most plentiful. Cr^{III} is found naturally in the environment, whereas Cr^{VI} is normally produced by industrial processes. The Cr^{III} generally occurs in the form of chromite ($FeOCr_2O_3$), however Cr^{VI} occurs in association with oxygen forming chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$), all these forms are toxic to all life forms (Ertani *et al*., 2017). The most stable form of chromium is Cr^{III} depending on its activity, while the form Cr^{VI} is the highest poisonous one which greatly hindering plant growth due to their enhanced solubility and higher oxidizing potential (Pawlisz *et al*., 1997).

It is a known fact that vegetables being a major dietary source of essential nutrients for human, are consumed worldwide. However, in the present scenario of escalating environmental pollution due to expanding industrialization, the contamination of agricultural soil due to heavy metals is a major environmental issue. Due to the critical adverse ecological consequences and severe and persistent toxic effects of heavy metals on plant community, the growth, yield and quality of vegetables are being greatly affected. The plant community when exposed to environmental stress like heavy metal toxicity, they themselves would produce many dynamic adjustments and responses in their biological characteristics in order to cope up with such contaminations and thereby arising environmental situations for the survival. This may include various changes in morphological and anatomical structures, changes in the mechanisms of physiology and metabolism and even genetic characteristics (Nikolić *et al*., 2017).

Punz and Sieghardt in 1993 observed that plants growing in heavy metal polluted soil have decreased cell-wall component synthesis, hampering of mitosis, golgi apparatus damage and variations in polysaccharide metabolism within the root cells. Another

critical effect of heavy metal stress can be reduced root area in plants which may decrease their ability to search for water available in the soil causing deficiency in taking up water and mineral nutrients thereby developing shoot impairment. Oxidative damage induced by heavy metal can disrupt the usual mechanism of judicious uptake of selective inorganic nutrients. This may result in the passive entry of large amount of heavy metal into the root and from there to the shoot system resulting in oxidative damage to photosynthetic and mitochondrial apparatus. These impacts of heavy metals can induce diminished shoot growth and inferior root development causing poor morphological growth performance, reduced biomass and productivity (Bishnoi *et al.,* 1993; Gbaruko & Friday, 2007). Reports on the harmful effects of heavy metals on both metabolic and biochemical activities including reduced uptake of nutrients (Ghnaya *et al*., 2007), variations in nitrogen metabolism (Wang *et al*., 2007), its intervention in protein and carbohydrate metabolism, alterations in water balance and hinderance in stomatal opening and conductance are available (Sandalio *et al*., 2001; Djebali *et al*., 2010). Metal toxicity of any kind is attributed to an intricate sequence of interactions with signal transduction, cellular macromolecules and genetic processes (Santos & Rodriguez, 2012). There are certain literatures and observations available by researchers that the oxidative stress induced by heavy metals and other extreme stressful environmental conditions can deteriorate nutritional value of crop plants due to alterations in the structural and functional properties of nutrients and vitamins (Pleasants *et al*. 1992). Khanna-Chopra (2012) reported degradation in nutritional quality of crop plants under metal toxicity induced damage to chloroplast which leading to lipid peroxidation. Similarly, there are reports indicate that the reactive oxygen species production induced by oxidative stress under heavy metal toxicity in plants are capable of causing cytotoxic and genotoxic effects which may results in damage to various cellular components, genome instability and abnormal gene expression and which in turn may cause physiological and metabolic imbalances (Odeigah *et al*., 1997; Britt, 1999; Dovgaliuk *et al*., 2001; Subhajit *et al*., 2018). Further, some literatures cited that the excess accumulation of heavy metals and other toxins in plants growing under polluted environment is susceptible to undesirable changes and modifications in anatomy causing reduced growth and biomass (Zhang *et al.,* 2009; Rahoui *et al.,* 2010; Balaji *et al*., 2011).

All the undesirable impacts of oxidative damage under environmental pollution and particularly heavy metal stress and toxicity have been reported to have serious agricultural and environmental issue limiting crop production and quality. Further, the vegetables we intake are usually strong accumulators of heavy metals thereby posing high risk to our health too. It is estimated that 90% of the total metal intake in humans is via vegetables and fruits consumption and the rest 10% occurs through dermal contacts and inhalation of metal contaminated dust (Martorell *et al*. 2011; Khan *et al*. 2014). In the light of the above facts, assessment of the impact of heavy metal stress on various growth parameters of agricultural plants like physiology and metabolism, morphology and anatomy, genetical and cytological characteristics, biomass production and quality have gained empirical significance leading to momentum in relevant research.

RESEARCH PROBLEM AND OBJECTIVE OF THE STUDY

As identified by research, a diet rich in vegetables and fruits have many health benefits. It can lower blood pressure, reduce the risk of heart diseases, prevent some types of cancer, lower risk of eye and digestive problems, and have a positive effect upon blood sugar level. These benefits have made green leafy vegetables an excellent component of the habitual diet in most of the Tropical countries. Among all leafy vegetables, Amaranth is one which is easily cultivable and requires less attention and expenses. Though rich in minerals, vitamins and essential micronutrients, it is the cheapest leafy vegetable available in the market and is often referred to as "Poor man's vegetable". One of the major environmental problems which hinders crop productivity and quality in agricultural sector is heavy metal stress impacted by enormous increase in industrialization. This situation is alarming in developing countries where untreated heavy metal containing wastewater from industries is extensively used for irrigation or is disposed off carelessly into open environments. Among the different types of heavy metals, chromium deserves a notable attention due to its potential hazards on biological community (WHO, 1989). Heavy metal chromium is identified as a non-essential element which does not have any specific biological role in plant physiology and metabolism (Dixit *et al*., 2002; Reale, 2016). It is generally understood that excessive Cr levels in plant tissue is toxic and it may evoke several morpho-physiological and biochemical processes which can alter the growth and development, productivity and quality (UdDin *et al*., 2015; Kamran *et al*., 2017). Besides, it is identified that the leafy vegetables generally tended to accumulate excessive levels of heavy metals in the roots, stem and leaves compared to other vegetable crops (Yargholi & Azimi, 2008). Being non- degradable in nature and readily taken up by crop plants, the metal pollutants are likely to enter easily into the food chain causing serious health hazards to human beings.

Among the various forms of chromium, Hexavalent chromium (Cr^{VI}) is considered more toxic being a strong oxidant with a high redox potential resulting in a faster and excessive generation of reactive oxygen species (ROS). The oxidative stress induced generation of ROS can disturb the redox balance thereby affecting the water relations and physiology of plants leading to inhibition of growth performance (Anjum *et al*., 2017). Cr^{VI} finds

many industrial applications in steel production, electroplating, leather tanning, metal finishing, metal corrosion inhibition, textile paints and pigment manufacture, catalysts application, drilling mud, fungicides and nuclear weapons production. Therefore, Cr^{VI} metal finds its presence in the effluents of these industries as well as in municipal sewage (Zayed & Terry, 2003). Irrational discharge of chromium rich effluents eventually result in significant increase in Cr content in the environment (Joutey *et al*., 2015) and at present it is the second most common heavy-metal contaminant of groundwater, soil and sediments (Srivastava & Thakur, 2006). Cr has been ranked 17^{th} position among the most hazardous substances (CERCLA Priority List of Hazardous Substances, 2017) by the Agency for Toxic Substances and Disease Registry and it has been rated as the number one carcinogen as per the International Agency for Research on Cancer (IARC, 1987). In view of the above facts, heavy metal toxicity in Amaranthus species, being a widely consumed leafy vegetable, is a matter of grave concern for research. Nevertheless, the impact of Cr toxicity has not acquired the required attention from the scientific and research community compared to other toxic metals like cadmium, mercury and lead in spite of the fact that the increased bioavailability of hexavalent chromium (Cr^{VI}) in the agricultural land is, no doubt, a major threat to productivity and quality of crop plants. Considering all the above facts, the present study is carried out with the following objectives to evaluate the impact of different levels of Hexavalent chromium (Cr^{VI}) stress and toxicity on the growth performance, biomass yield and quality of *Amaranthus dubius* Mart. ex Thell., a cultivable crop species (CO1) of Amaranthus which is best suited for tropical humid regions.

a) Impacts of hexavalent chromium (Cr^{VI}) stress on Morphology and Growth and Biomass production
b) Assessment of toxicity level in terms of Tolerance Index (TI in %) and Inhibition of Plant Growth (IPG in %)
c) Assessment of Physiological and Biochemical alterations induced under hexavalent Chromium (Cr^{VI}) stress
d) Impact of hexavalent chromium (Cr^{VI}) stress on Anatomical parameters
e) Geno-Cytotoxic effects of hexavalent chromium (Cr^{VI}) stress

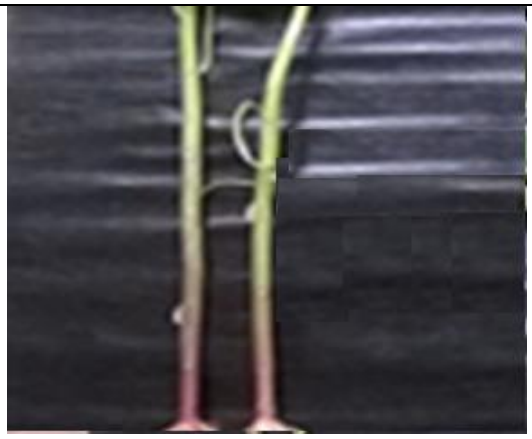
f) Impact of hexavalent chromium (Cr^{VI}) stress on Macro and Micronutrient elemental uptake and balance
g) Assessment on the impact of hexavalent chromium (Cr^{VI}) stress on Nutritional Value
h) Analysis in the Differential Expression of Genes (DEGs) under hexavalent chromium (Cr^{VI}) stress to reveal molecular level changes

Amaranthus dubius is an erect, annual herb belongs to the family Amaranthaceae, growing up to an average of 90cm tall. The leaves are simple having ovate to rhomboid-ovate shaped lamina with long petiole arranged spirally around the stem. The stem is slightly stout, little or unbranched, green in colour in the top region and very often pinkish red in color towards the bottom region. Flowers are small, green. They are grouped in small dense balls spread along slender spikes at the base of the leaves and the top of the stems. The fruit is a small capsule that opens at maturity by a small cap at the end. It contains a single shiny seed, dark brown to black in color.

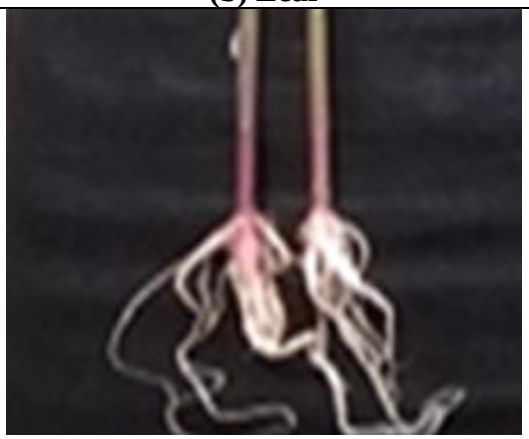
The taxonomic identity of the plant *Amaranthus dubius* was confirmed with the Department of Botany, Sree Narayana Mangalam College, Maliankara, Ernakulam, Kerala and the voucher specimens (SNMH- 6894) of the plant has been deposited in the Herbarium collection of the Dept. of Botany, SNM College (NBG Accredited) for further reference.

(a) Habit

(b) Leaf



(c) Stem



(d) Root

(e) Inflorescence

Figure 1.1: Photographs showing the characteristics of *Amaranthus dubius* Mart. ex Thell.

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CHAPTER-2

IMPACTS OF HEXAVALENT CHROMIUM (Cr^{VI}) STRESS ON MORPHOLOGICAL GROWTH PARAMETERS AND BIOMASS PRODUCTION

INTRODUCTION

In the recent years, the type and quantity of heavy metal deposition in the agricultural soil has enhanced multifold due to impact of industrialization and geo chemical activities. This has been an area of great concern as it adversely affects the environment and organisms alike. Among the heavy metal deposition, Chromium (Cr), due to its non-biodegradable nature affects plant growth and productivity (Singh *et al*., 2013; Dotaniya *et al*., 2014; Samantaray *et al*., 2015). Chromium ions which are almost insoluble in soil, when available in higher concentrations, can built up a conclusively toxic environment for plant growth (Srivastava & Thakur, 2006). When absorbed by plants in excess concentrations, the critical effect of chromium and likewise heavy metal stress can be decreased root area in plants, which may reduce their capacity to search for water and mineral nutrients available in the soil. This may cause deficiency of water and mineral nutrients essential for various plant growth performances (Bishnoi *et al.*, 1993; Gbaruko & Friday, 2007). It is identified as a serious agricultural and environmental issue limiting crop production.

Among the various inhibitory responses identified in response to environmental stress like heavy metal contamination, the morphological growth changes are the most natural and visually observable variations occurring in plants during the struggle and the fight against such abiotic stress. The studies on the toxic effects of heavy metals on plant growth, it is understood to have alterations in the germination process as well as in the growth of roots, stems and leaves. Hence, vulnerability to different levels of Cr stress may causes deterioration in total fresh as well as dry matter production and yield (Shanker *et al*., 2005). Tang *et al*. in 2001 identified that of the different morphological parameters in plants, the root growth is most commonly affected due to heavy metal

stress and this fact is very well documented in both trees and crops. The deteriorated root growth owing to the availability of heavy metals like Cr may be caused by root cell inhibition or elongation which may be due to tissue collapse and subsequent inability of roots for water and mineral absorption (Zou *et al*., 2006), together with retardation in cell cycle processes (Sundaramoorthy *et al*., 2010). The transportation of heavy metals like chromium into the aerial parts of the plant can have a direct influence on the cellular metabolism of shoot resulting in reduced height and growth (Oliveira, 2012). Expansion of leaf area is another noticeable morphological characteristic when subjected to stressful environment specifically to heavy metals. Pandey *et al*. (2005) reported a decrease in leaf area as one of the significant symptoms in *Brassica juncea* on exposure to heavy metal toxicity and suggested it to be an indicator of heavy metal contamination. In general, the major processes involved in the leaf area expansion and plant growth are cell division and enlargement. Reduction in cell number and cell size of the leaves are the contributing factors for decreased leaf area due to metal toxicity (Nieman, 1965). The negative effects on hydraulic conductance and water potential due to oxidative stress induced by metal toxicity and subsequent restrictions in the intake of vital nutrients by plant tissues may disrupt the process of leaf area expansion and growth (Hall, 2002; Poschenrieder & Barceló, 2004; Nagajyoti *et al*, 2010; Marschner, 2011). It is explicitly clear that the tremendous progress in industrialization as contributed to escalating heavy metal soil contamination, especially by way of metal chromium. This has a disastrous impact on the productivity of agricultural crops which subsequently may cause food scarcity in the coming years. Considering all the above aspects, the study undertaken in *Amaranthus dubius*, the poor man's vegetable, has appreciable relevance. This chapter tries to assess the effect of different concentration levels of hexavalent chromium (Cr^{VI}) stress on various morphological growth parameters and productivity in terms of biomass accumulation. The study also attempts to evaluate the tolerance level and growth inhibition of *A. dubius* to Cr^{VI} stress.

REVIEW OF LITERATURE

Heavy metals, especially Cr are predominant environmental contaminants which are closely observed by the scientific community due to their acute toxicity and relatively undetected mode of action. In the recent decades their excessive levels in agricultural soils and ground water due to a variety of anthropogenic activities is a cause of serious risk not only for normal plant growth and crop yield but also for the human and animal health (Gill *et al.* 2015, Shanker *et al*. 2005). Retarded plant growth, chlorosis in new green leaves, imbalance in nutrients , wilting of tops, and injurious roots are all outcomes of excess chromium (Chatterjee & Chatterjee, 2000, Dixit *et al*., 2002). Sharma *et al*. in 2003 and Scoccianti *et al*. in 2006 have reported that hampered plant growth owing to chlorosis in young leaves, impairment in photosynthesis, drooping of tops and root damage are attributed to the inducement and development of reactive oxygen species (ROS) under Cr toxicity. According to Zhao *et al*. (2019), Cr stress causes a reduction in the availability of important nutrients like sodium, iron, manganese, copper, zinc, and calcium imparting a negative effect on the vital metabolic activities of plants resulting in substantial growth and yield decrement. The production of biomolecules via lipids, nucleic acids and proteins are impaired due to ROS generation induced by Cr stress as a result of which the mitochondrial respiration and carbohydrate metabolism are interrupted causing a negative influence on growth and development. (Gill & Tuteja, 2010, Gill *et al*., 2015). Samantaray *et al*. (1996) is reported to have identified drastic chlorosis & necrosis in leaves and growth stunting in *Oryza sativa* and *Echinochloa colona* plants which are grown in soil with excess concentration of chromium content.

Several symptoms of morphological stress are noticed in plants grown in soils with excessive heavy metal contamination. Peralta and coworkers in 2001 showed that $5mgL^{-1}$ of Cr^{VI} caused an increase in root growth compared to control whereas higher doses (20 and $40mgL^{-1}$) indicated an inhibition effect. This retardation in root and shoot lengths at higher dosage of chromium may be due to the negative impact of this metal on auxin synthesis (Ghani & Ghani, 2011). Hunter & Vergnano (1953) also reported severe stunted growth, poor root development and chlorosis of leaves in Oats plant due to chromium toxicity. When treated with Cr^{V1}, the roots of *Zea mays* L. grew shorter and

brownish with a smaller number of roots hairs (Mallick *et al*., 2010). Sensitive mungbean cultivars also exhibited reduced root growth on exposure to Cr^{VI} (Rout *et al*., 1997). *Triticum aestivum* L. seedlings when exposed to $100mgL^{-1}$ of Cr^{VI}. Dey and coworkers (2009) established a decrease in root length by 63% and shoot length by 44%, compared to control. Mallick and coworkers (2010) observed that shoot length of *Zea mays* L. decreased significantly after Cr^{VI} treatment. Rout and coworkers (2000) identified a reduction in plant height and shoot growth on Cr exposure in sensitive mungbean plants.

Reduction in total leaf area, leaf number and size are exhibited by plants grown under heavy metal stress. Buendia- Gonzalez *et al*. (2010) observed a decrease in number of leaves in *Prosopis laevigata* under Cr^{VI} stress. Hydraulic conductance and water potential of plant tissues may be affected adversely by high content of Cr^{V1} may also contribute to reduce the leaf area and shoot growth under heavy metal treatment (Poschenrieder & Barceló, 2004). As a result of heavy metal application, there may be reduction in the intake of major nutrient elements such as potassium and nitrate causing reduced leaf area (Hall, 2002; Nagajyoti *et al*, 2010; Seregin & Ivanov, 2001).

The plant growth retardation caused by metal stress is due to lower water potential and nutrient uptake combined with alterations in the functioning of many key enzymes of different pathways (Arduini *et al*., 1996) along with disturbed microtubule organization in meristematic cells (Eun *et al*., 2000). Toxicity of heavy metals is more predominant in roots as they are accumulated there effecting retarded cell division and elongation (kidd *et al*., 2004). Roots get more affected compared to shoots, may be due to interference of metal chromium with auxin regulated cell elongation (Ernst *et al.*, 1992). Stimulation of 1AA oxidation is induced as a result of heavy metal stress which lead to auxin deficiency thereby restricting cell growth (Mukherjee & Roy, 1977). Ultrastructural changes in chloroplasts are caused by Cr toxicity which is evident in the form of poorly developed lamellar system with widely spaced thylakoids and fewer grana (Ali *et al*., 2013). This change may adversely affect CO_2 fixation, electron transport, photophosphorylation, and enzyme activities of the Calvin cycle causing inhibition of photosynthesis thereby leading to poor growth (Ali *et al*., 2013; Gill *et al*., 2015). Metal tolerance assessment is an area of prime importance while selecting plants for utilization in phytoremediation (Zacchini

et al., 2009). In order to characterize metal tolerance in plants, one of the most usual parameters used is the tolerance index (TI) (Köhl & Lösch, 1999).

MATERIALS AND METHODS

Collection and sterilization of seeds:

Healthy seeds of *Amaranthus dubius are collected from* Kerala Agricultural University, Thrissur. The seeds are surface sterilized in 0.5 % sodium hypochlorite solution for 20 minutes and washed thoroughly with distilled water to prevent any fungal contamination.

Preparation of growing media and heavy metal concentrations

Growing media for the post germination treatment study is standard potting mixture prepared in the ratio of 1:1:1 which contains Farmyard manure, Red soil and Sand respectively. Different concentrations of heavy metal hexavalent chromium (Cr^{VI}) are prepared from Potassium dichromate salt ($K_2Cr_2O_7$) of analytical reagent grade.

Preliminary germination study

Surface sterilized healthy seeds of *Amaranthus dubius* plants are subjected to germination under different concentrations of metal hexavalent chromium (Cr^{VI}) like 1mg, 5mg, 10mg, 20mg, 40mg, 80mg 100mg Cr^{VI}/litre and 0mg Cr^{VI}/litre water as control have been used to check the performance of seed germination for selecting suitable test concentrations of Cr^{VI} for the post-germination treatment studies. The Final Germination Percentage (FGP %) described by Scott *et al.* (1984) and Germination Index (GI) described by Bench Arnold *et al.* (1991) are considered for the evaluation and selection of test concentrations.

Final Germination Percentage (FGP %) (Scott *et al*., 1984)

FGP = FGP=Final no. of seeds germinated in a seed lot × 100

The higher the FGP value, the greater the germination of a seed population.

Germination Index (GI) (Bench *et al*., 1991)

GI=(10×n1) + (9×n2) + · · · + (1×n10)

Where, n1, n2 . . . n10 = No. of germinated seeds on the first, second and subsequent days until the 10th day.

The digits 10, 9 . . . and 1 are weights given to the number of germinated seeds on the first, second and subsequent days, respectively.

Principle: In the GI, maximum weight is given to the seeds germinated on the first day and less to those germinated later on. The lowest weight would be for seeds germinated on the 10th day. Therefore, the GI emphasizes on both the percentage of germination and its speed. A higher GI value denotes a higher percentage and rate of germination.

Table 2.1: Preliminary seed germination study in *Amaranthus dubius* under different levels of Cr^{VI} stress

Treatments chromium (mg/L)	Number of seeds germinated in the day										Final Germination Percentage (FGP %)	Germination Index (GI)
	1	2	3	4	5	6	7	8	9	10		
1mg	0 0 0	0 0 0	33 30 28	22 21 19	5 4 3	2 2 2	0 0 0	0 0 0	0 0 0	0 0 0	**58.00** *±4.58*	**429.00** *±33.72*
5mg	0 0 0	0 0 0	34 28 22	13 18 23	4 2 0	5 2 2	0 0 0	0 0 0	0 0 0	0 0 0	**51.00** *±4.58*	**377.00** *±32.79*
10mg	0 0 0	0 0 0	31 26 21	15 16 17	4 4 4	1 2 3	0 0 0	0 0 0	0 0 0	0 0 0	**48.00** *±3.00*	**354.00** *±28.00*
20mg	0 0 0	0 0 0	17 14 11	4 5 6	17 17 13	1 0 1	0 0 1	0 0 0	0 0 0	0 0 0	**35.67** *±3.51*	**245.67** *±27.15*
40mg	0 0 0	0 0 0	9 7 7	3 2 3	10 10 6	4 4 4	0 0 0	0 0 0	0 0 0	0 0 0	**23.00** *±3.00*	**152.00** *±20.07*
80gm	0 0 0	0 0 0	2 0 1	2 2 2	9 9 6	2 1 1	0 0 0	0 0 0	0 0 0	0 0 0	**12.33** *±2.52*	**76.67** *±15.82*
100mg	0 0 0	0 0 0	0 0 1	2 1 2	3 6 6	1 1 1	0 0 0	0 0 0	0 0 0	0 0 0	**8.00** *±2.00*	**49.33** *±13.05*
Control	0 0 0	0 0 0	29 32 35	20 21 22	3 3 3	2 2 3	0 0 0	0 0 0	0 0 0	0 0 0	**58.67** *±4.04*	**435.00** *±30.20*

Table 2.2: Duncan analysis of final germination percent (FGP%) of *Amaranthus dubius* treated with Cr^{VI} stress

	Subset for alpha = 0.05				
Code	1	2	3	4	5
7 (80mg)	8.0000				
6 (40mg)	12.33				
5 (20mg)		23.00			
4 (10mg)			35.67		
3 (5mg)				48.00	
2 (1mg)				51.00	
1 (Control)					58.00
8 (100mg)					58.67
Sig.	.151	1.000	1.000	.312	.819

Means for groups in homogeneous subsets are displayed

Table 2.3: Duncan analysis of seed germination index (GI) in *Amaranthus dubius* treated with Cr^{VI} stress

	Subset for alpha = 0.05				
Code	1	2	3	4	5
7 (80mg)	49.33				
6 (40mg)	76.67				
5 (20mg)		152.00			
4 (10mg)			245.67		
3 (5mg)				354.00	
2 (1mg)				377.00	
1 (Control)					429.00
8 (100mg)					435.00
Sig.	.219	1.000	1.000	.297	.782

Means for groups in homogeneous subsets are displayed

The preliminary germination study clearly revealed inhibitory impact of hexavalent chromium (Cr^{VI}) stress on the germination of *A. dubius* plants (table 2.1). With an exception of initial level stress of 1mg Cr^{VI} concentration treatment which caused only slight inhibition of 0.66% seed germination, all other Cr^{VI} stress treatments have caused significant inhibition over the control with the highest inhibition of 92% has been recorded in the 100mg Cr^{VI} stress. The study revealed the intensity of inhibition is dose dependent and it is clearly evident from the progressive reduction in the final germination

percentage (FGP%) and the germination index (GI) recorded in progression with increase in Cr^{VI} concentration treatments (table 2.2 & 2.3). Based on the preliminary analysis, seven different concentrations of Cr^{VI} are selected for the post germination treatment study to evaluate the impact of different levels of Cr^{VI} stress on various morphological growth and biomass production parameters in *A. dubius*. The selected test concentrations are 1mg, 5mg, 10mg, 20mg, 30mg, 50mg & 70mg Cr^{VI}/kg potting mixture. 0mg Cr^{VI}/kg potting mixture taken as control.

Post Germination Treatment Study: Assessment of Morphological Growth Parameters

The germinated seeds which emerge out of the growing media surface are transferred carefully into plastic pots containing standard potting mixture. One week after transplantation, healthy uniform sized potted seedlings in triplicate blocks of 30 each are arranged for treatment with different specific concentrations of 1mg, 5mg, 10mg, 20mg, 30mg, 50mg & 70mg of chromium (Cr^{VI}) /kg potting mixture. The required concentrations of chromium are applied to the potting mixture as aqueous solution in such a way that every 200ml contains required quantities of chromium. 200ml of distilled water alone is used as control (0.0mg/200ml). The evaluation of the following morphological growth parameters is carried out on the 40^{th} day of Cr^{VI} treatments.

a) Root length

b) Shoot length

c)Total plant length

d)Number of leaves

e) Fresh and Dry biomass of Shoot, Root and Total plants

Estimation of dry biomass by placing in an oven at 80°C for 24hours. Seedling fresh and dry biomass is measured by using electronic balance.

f) Root/Shoot ratio

g) Dry wit/Fresh wit ratio

h) Leaf area (Leaf area measurements are done using leaf area meter

(Model LI- 3100C, LI-COR Biosciences, USA).

i)The tolerance index (TI) (Wilkins, 1978)

TI (%) = 100 x (root length in metal treatment)/ (root length in the control)

j) Inhibition of seedling growth (Chou & Muller, 1972)

$$ISG\ (\%) = \frac{\text{Length of control seedling} - \text{Length of test seedling}}{\text{Length of control}} \times 100$$

Statistical Analysis

The mean value and standard deviation of various parameters in the study are determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSION

Shoot, Root and Total Plant Length

The effect of different concentrations of hexavalent chromium (Cr^{VI}) on the shoot length, root length and total plant length of *Amaranthus dubius* on the 40th day after treatments is depicted in the table 2.4. The data shows shoot length of all Cr^{VI} treated plants recorded a reduction progressively over the control plants along with the increase in the concentration of treatments. The highest reduction of 74.66% of shoot length is recorded in the treatment of 70mg Cr^{VI}/kg soil while the lowest reduction of 2.53% is obtained with 1mg Cr^{VI}/kg soil treatment. With respect to the length of root and total plant, with an exception of lowest Cr^{VI} concentration treated plants of 1mg Cr^{VI}/kg soil, all the concentration treatments exhibited a similar trend as in the case of shoot length while the lowest concentration caused an enhancement of 14.55% and 4.22% for root and total plant length respectively over the control plants. The highest reduction of 86.42% for root length and 78.83% for total plant length is obtained with 70mg Cr^{VI}/kg soil treatment while the lowest reduction of 68.09% and 51.36% respectively for root and total plant length is obtained with 5mg Cr^{VI}/kg soil treatment (figure 2.1 & 2.1a).

Table 2.4: Effects of different concentrations of Cr^{VI} stress on *Amaranthus dubius* shoot, root and total plant length at 40th day after treatment

Treatment Cr^{VI} / kg soil	**Length in cm**					
	Shoot	SD ±	**Root**	SD ±	**Total plant**	SD±
1 (Control)	51.33	8.9	28.17	3.69	79.50	8.2
2 (1mg)	50.00	5.3	33.00	2.5	83.00	7.8
3 (5mg)	29.67	3.4	9.00	1.3	38.67	3.5
4 (10mg)	24.17	3.8	7.50	1.5	31.67	5.2
5 (20mg)	22.43	1.8	7.67	0.76	30.10	1.3
6 (30mg)	17.33	1.3	7.00	0.87	23.67	2.3
7 (50mg)	15.67	1.9	5.00	1.0	20.67	2.8
8 (70mg)	13.00	1.3	3.83	0.76	16.83	1.8

The statistical analysis revealed the differences in the shoot length and total plant length of control plants over the lowest concentration of 1mg Cr^{VI}/kg soil treated plants are not significant but their values differed significantly higher (0.05 level) over the values of rest of the treated plants. Similarly, the lowest shoot and total plant length obtained in plants treated with 70mg Cr^{VI}/kg soil did not differ significantly over the values of plants treated with 50mg and 30mg Cr^{VI}/kg soil but their differences with control and all other treated plants are significantly lower (table 2.5 & 2.7). The highest root length obtained in plants treated with 1mg Cr^{VI} is significantly higher over control and other treated plants while the lowest root length obtained with 70mg Cr^{VI} did not differ significantly over plants treated with 50 mg 30mg Cr^{VI} but differed significantly lower compared to rest of the treated plants (table 2.6).

Table 2.5: Duncan analysis of shoot length variation in *Amaranthus dubius* treated with Cr^{VI}

	Subset for alpha = 0.05				
Treatment	1	2	3	4	5
8 (70mg)	13.00				
7 (50mg)	15.67	15.67			
6 (30mg)	17.33	17.33	17.33		
5 (20mg)		22.43	22.43	22.43	
4 (10mg)			24.17	24.17	
3 (5mg)				29.67	
2 (1mg)					50.00
1 (Control)					51.33
Sig.	.253	.081	.079	.064	.705

Means for groups in homogeneous subsets are displayed

Table 2.6: Duncan analysis of root length variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	3.83				
7 (50mg)	5.00	5.00			
6 (30mg)	7.00	7.00	7.00		
4 (10mg)		7.5.	7.5		
5 (20mg)			7.67		
3 (5mg)			9.00		
1 (Control)				28.17	
2 (1mg)					33.00
Sig.	.060	.119	.235	1.00	1.00

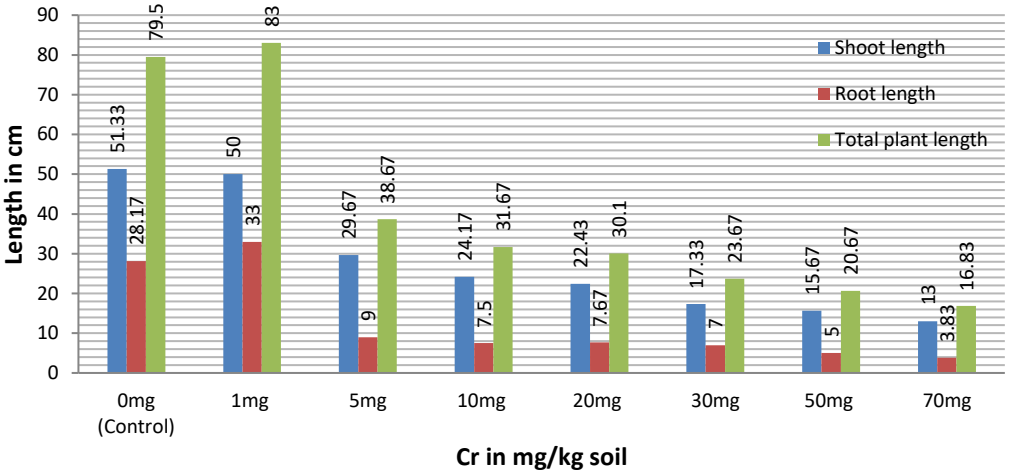
Means for groups in homogeneous subsets are displayed

Table 2.7: Duncan analysis of total plant length variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	16.83			
7 (50mg)	20.67			
6 (30mg)	23.67	23.67		
5 (20mg)		30.10	30.10	
4 (10mg)		31.67	31.67	
3 (5mg)			38.67	
1 (Control)				79.50
2 (1mg)				83.00
Sig.	.117	.070	.054	.385

Means for groups in homogeneous subsets are displayed

Figure 2.1: Impacts of different concentrations of Cr^{VI} on *Amaranthus dubius* shoot, root and total plant length



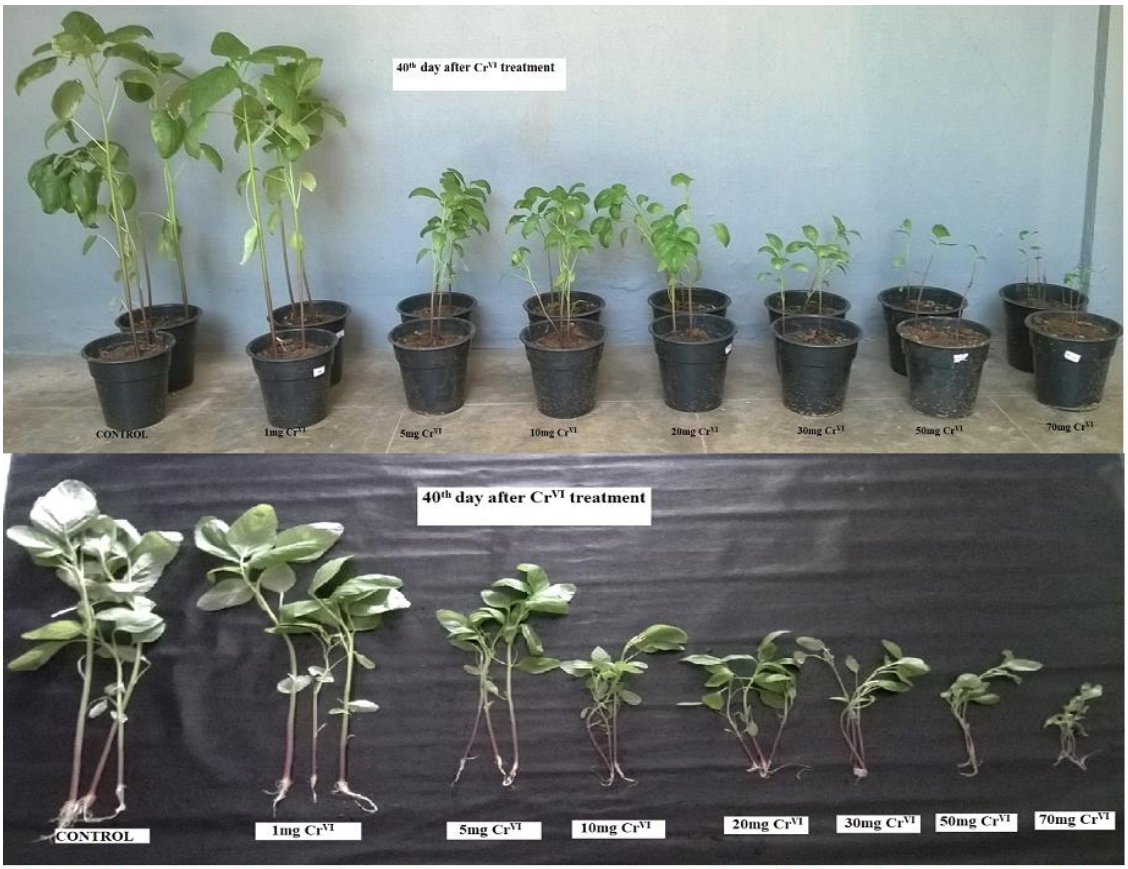


Figure 2.1a: Morphological growth variations in *Amaranthus dubius* plants treated with different concentration stress of hexavalent chromium (Cr^{VI}) over the control

The root is the part of the plant which is in direct contact with hexavalent chromium (Cr^{VI}) available in the growing medium. Cr^{VI} enters the root system along with carrier elements like sulphate and iron and accumulates in higher concentration in the

root component compared to other regions of the plant due to immobilization of chromium in vacuoles of root cells (Oliveira, 2012; Nematshahi *et al*., 2012; Kumar & Maiti, 2013). The toxic concentration of chromium in the root cells may disturb cell division and elongation and can cause imbalances in Ca^{2+} transport between plasma membrane and cytoplasm (Zou *et al*., 2006). This might be the reason that contributed towards the reduction in length and growth of root under chromium stress in *A. dubius* plants. Besides, the reduced root growth can also be attributed to toxicity induced elongation of cell cycle in the roots (Srivastava & Jain, 2011). The chromium toxicity induced inhibition of shoot length and growth is observed and reported previously by several workers (Sharma & Sharma,1993; Barton *et al*.,2000) and the present study inferences are also in similar lines with these reports. The reduced uptake of water and minerals and their poor mobilization to shoot system imposed by poor root system development, root injury and suberization of root under chromium stress could be the result of reduced shoot growth performances over control plants (Davis, 1986; Breckle, 1991; Samantaray & Das, 1997; Samantaray *et al*., 1998; Saddiqe *et al*., 2015).

Fresh and Dry weight Biomass of Shoot, Root and Total Plant

The various concentrations of hexavalent chromium (Cr^{VI}) treatments in *Amaranthus dubius* plants caused differences in the fresh weight of shoot and root components and in the total plant over control. The details are depicted in the table 2.8. **A**ll the Cr^{VI} treated plants displayed a decrease in fresh weight progressively over the control plants as the concentration of treatment is increasing. The highest decrease of 97.08% shoot fresh weight is recorded in the treatment of 70mg Cr^{VI}/kg soil while the lowest decrease of 10.53% recorded in 1mg Cr/kg soil treatment. A similar trend is also recorded with respect to fresh weight of root component and total plant. The highest decrease of 97.66% for root component and 97.16% for total plant over control is obtained with 70mg Cr^{VI}/kg soil treatment while the lowest decrease of 14.06% and 10.99% respectively for root component and total plant is obtained with 1mg Cr^{VI}/kg soil treatment (figure 2.2).

Table 2.8: Effects of different concentrations of Cr^{VI} stress bon **fresh weight** of shoot, root and total plant in *Amaranthus dubius* at 40th day after treatment

Treatment Code Cr^{VI} / kg soil	Fresh weight (FW) in g					
	Shoot	SD ±	Root	SD ±	Total plant	SD ±
1 (Control)	33.90	4.8	5.12	0.94	39.02	5.7
2 (1mg)	30.33	5.2	4.40	0.53	34.73	5.7
3 (5mg)	6.37	1.0	0.52	0.13	6.89	1.1
4 (10mg)	4.53	2.1	0.35	0.13	4.88	2.3
5 (20mg)	4.45	1.2	0.31	0.17	4.76	1.4
6 (30mg)	3.43	0.82	0.25	0.06	3.68	0.87
7 (50mg)	2.15	0.15	0.18	0.03	2.35	0.20
8 (70mg)	0.99	0.15	0.12	0.02	1.11	0.17

The differences in the fresh biomass accumulation of shoot component as well as the total plant between the control plants and plants treated with lowest concentration of 1mg Cr^{VI} are not significant but their values differed significantly higher (0.05 level) over the values of rest of the treated plants (table 2.9 & 2.11) while the decrease in the root fresh biomass in all the Cr^{VI} treated plants are significantly lower over control (table 2.10). The lowest fresh biomass values of shoot component are recorded by 70mg Cr^{VI} treated plants which differed significantly lower over the values of control and plants treated with 1mg and 5mg Cr^{VI} while the same for the fresh biomass of root component as well as the total plant have their values differing significantly lower only over control and 1mg Cr^{VI} treated plants.

Table 2.9: Duncan analysis of shoot fresh weight variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	.99		
7 (50mg)	2.15	2.15	
6 (30mg)	3.43	3.43	
5 (20mg)	4.45	4.45	
4 (10mg)	4.53	4.53	
3 (5mg)		6.37	
2 (1mg)			30.33
1 (Control)			33.90
Sig.	.162	.099	.122

Means for groups in homogeneous subsets are displayed.

Table 2.10: Duncan analysis of root fresh weight variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	.12		
7 (50mg)	.18		
6 (30mg)	.25		
5 (20mg)	.31		
4 (10mg)	.35		
3 (5mg)	.52		
2 (1mg)		4.40	
1 (Control)			5.12
Sig.	.282	1.000	1.000

Means for groups in homogeneous subsets are displayed

Table 2.11: Duncan analysis of total plant fresh weight variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05	
	1	2
8 (70mg)	1.11	
7 (50mg)	2.35	
6 (30mg)	3.68	
5 (20mg)	4.76	
4 (10mg)	4.88	
3 (5mg)	6.89	
2 (1mg)		34.73
1 (Control)		39.02
Sig.	.053	.103

Means for groups in homogeneous subsets are displayed.

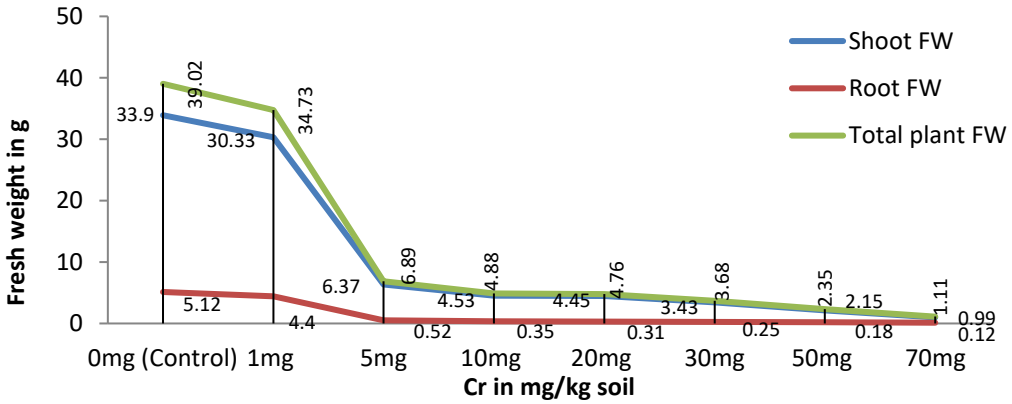


Figure 2.2: Effects of different concentrations of Cr^{VI} on the fresh weight of *Amaranthus dubius* shoot, root and total plant

Similarly, the Cr^{VI} treatments also caused differences in the dry biomass of shoot and root components and in the total plant in *A. dubius* over control plants (table 2.12). The dry biomass of all the Cr^{VI} treated plants recorded a progressive reduction over the control plants as the concentration of Cr^{VI} treatment is enhancing. The highest reduction of 98.19%, 97.96% and 98.16% is recorded in the 70mg Cr^{VI}/kg soil treatment respectively for shoot, root and total plant while the lowest reduction of 9.99%, 14.74% and 10.52% is recorded in 1mg Cr^{VI} treatment respectively for shoot, root and total plant (figure 2.3).

Table 2.12: Effects of different concentrations of Cr^{VI} stress on **dry weight** of shoot, root and total plant in *Amaranthus dubius* at 40th day after treatment

Treatment code Cr^{VI} / kg soil	Dry weight (FW) in g					
	Shoot	SD±	Root	SD±	Total plant	SD±
1 (Control)	3.313	0.495	0.441	0.086	3.753	0.580
2 (1mg)	2.982	0.526	0.376	0.042	3.358	0.567
3 (5mg)	0.551	0.078	0.035	0.009	0.586	0.086
4 (10mg)	0.303	0.140	0.019	0.008	0.323	0.148
5 (20mg)	0.294	0.088	0.016	0.009	0.310	0.096
6 (30mg)	0.192	0.043	0.015	0.004	0.207	0.046
7 (50mg)	0.129	0.010	0.013	0.003	0.142	0.012
8 (70mg)	0.060	0.008	0.009	0.002	0.069	0.010

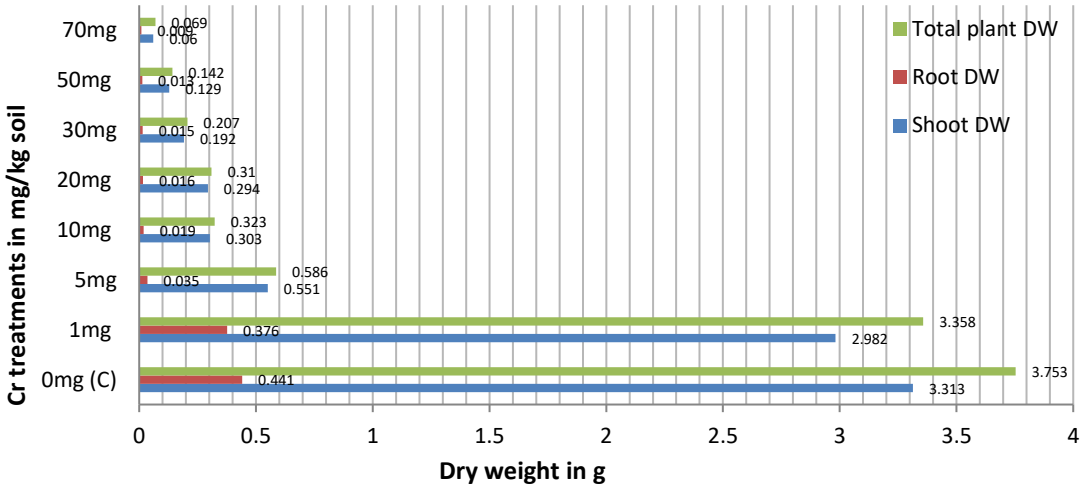


Figure 2.3: Effects of different concentrations of Cr^{VI} on the Dry weight of *Amaranthus dubius*

The reduction in dry biomass of shoot component and total plant in 1mg Cr^{VI} treated plants showed no significant differences over control while all other treated plants have recorded significant reductions in dry biomass over control as well as 1mg Cr^{VI} treated plants (table 2.13 & 2.15), whereas the reduction in root component in all the treated plants are significant over control (table 2.14). The highest decrement in dry biomass is recorded by 70mg Cr^{VI} treatment which showed no significant differences over 50mg, 30mg, 20mg, 10mg and 5mg Cr^{VI} treatments.

Table 2.13: Duncan analysis of **shoot dry weight** variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05	
	1	2
8 (70mg)	.0597	
7 (50mg)	.1290	
6 (30mg)	.1917	
5 (20mg)	.2943	
4 (10mg)	.3033	
3 (5mg)	.5510	
2 (1mg)		2.9820
1 (Control)		3.3113
Sig.	.058	.146

Means for groups in homogeneous subsets are displayed

Table 2.14: Duncan analysis of **root dry weight** variation in *Amaranthus dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	.009		
7 (50mg)	.013		
6 (30mg)	.015		
5 (20mg)	.016		
4 (10mg)	.019		
3 (5mg)	.035		
2 (1mg)		.376	
1 (Control)			.441
Sig.	.418	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 2.15: Duncan analysis of **total plant dry weight** variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05	
	1	2
8 (70mg)	.0687	
7 (50mg)	.1423	
6 (30mg)	.2067	
5 (20mg)	.3103	
4 (10mg)	.3227	
3 (5mg)	.5860	
2 (1mg)		3.3580
1 (Control)		3.7527
Sig.	.073	.122

Means for groups in homogeneous subsets are displayed.

The Cr^{VI} toxicity induced reduction in photosynthetic pigments and availability of essential nutrients like Zn, Mn, Cu, Fe, Na, Ca etc. is followed by the decreased shoot and root growth rate due to extended cell cycle and disturbed cell division (Zou *et al*., 2006; Srivastava & Jain, 2011) which in turn results in corresponding reduction in productivity, it might be the major factor responsible for the decreased fresh and dry biomass of shoot, root and total plant of *A. dubius* in the present study (Singh *et al*., 2013; Dotaniya *et al*., 2014; Zhao *et al*., 2019).

Root/Shoot weight ratio

The details of root/shoot weight ratio obtained in *A. dubius* plants treated with various concentrations of Cr^{VI} stress is shown in table 2.16. All the treatments have caused a reduction in root/ shoot ratio in terms of both fresh weight and dry weight over control, with an exception in the case of 70mg Cr^{VI}/kg soil treatment, where the root/shoot dry weight ratio recorded an insignificant increase over control. The results generally indicate the reductions in the ratio are progressive up to 20mg Cr/kg soil treatment and then indicate a progressive enhancement up to 70mg Cr^{VI}/kg soil treatment. The highest reduction of 55.61% of fresh weight ratio and 60.29% of dry weight ratio is recorded in 20mg Cr^{VI} treatment while the lowest reduction of 2.99% for fresh weight and 4.14% for dry weight is recorded in 1mg Cr^{VI} treatment. However, in the 70mg Cr^{VI} treated plants, R/S dry weight ratio recorded an increase of 11.36% over control (figure 2.4).

Table 2.16: Effects of different concentrations of Cr^{VI} stress on Root/Shoot weight ratio in *Amaranthus dubius* at 40th day after treatment

Treatment Code Cr^{VI} / kg soil	Root/Shoot ratio (R/S Ratio)			
	R/S fresh weight ratio	SD±	R/S dry weight ratio	SD±
1 (Control)	15.05	0.71	13.27	0.71
2 (1mg)	14.60	1.06	12.72	1.05
3 (5mg)	8.08	0.97	6.29	0.90
4 (10mg)	7.93	1.33	6.48	0.81
5 (20mg)	6.68	2.00	5.27	1.52
6 (30mg)	7.47	1.24	7.88	1.69
7 (50mg)	8.50	0.77	10.34	1.70
8 (70mg)	12.06	0.26	14.97	1.52

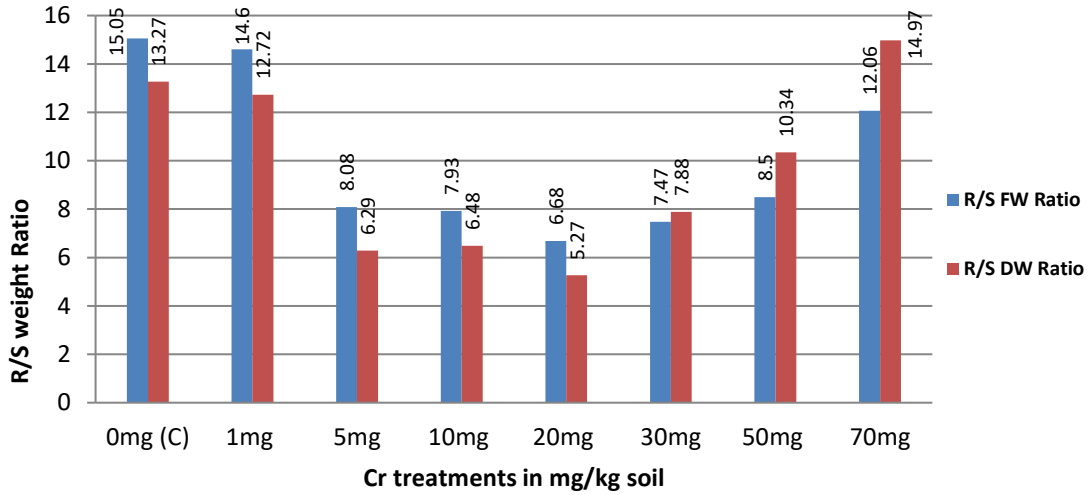


Figure 2.4: Effects of different concentrations of Cr^{VI} on Root/Shoot weight ratio in *A. dubius*

The decrease in R/S weight ratio of all the treated plants, with an exception in 1mg Cr^{VI} treated plants with respect to R/S fresh weight ratio and 1mg & 70mg Cr^{VI} treated plants with respect to R/S dry weight ratio, differed significantly lower over control (table 2.17 & 2.18). The differences in reduction of R/S fresh weight ratio between 5mg, 10mg, 20mg, 30mg and 50mg Cr^{VI} treated plants are insignificant while the 70mg Cr^{VI} treatment caused significant decrease over control as well as lowest concentration of 1mg Cr^{VI} treatment, but it differed significantly higher over the rest of the treatments. The decrease in R/S dry weight ratio caused due to 50mg Cr^{VI} treatment is significantly lower

over control, 1mg and 70mg Cr^{VI} treatments, but differed significantly higher over rest of the treatments.

Table 2.17: Duncan analysis of **R/S fresh weight ratio** variation in *A. dubius* treated with Cr^{VI}

Treatment Code	Subset for alpha = 0.05		
Cr^{VI} / kg soil	1	2	3
5 (20mg)	6.6788		
6 (30mg)	7.4734		
4 (10mg)	7.9274		
3 (5mg)	8.0787		
7 (50mg)	8.4981		
8 (70mg)		12.0612	
2 (1mg)			14.5968
1 (Control)			15.0467
Sig.	.098	1.000	.638

Means for groups in homogeneous subsets are displayed.

Table 2.18: Duncan analysis of **R/S dry weight ratio** variation in *A. dubius* treated with Cr^{VI}

Treatment Code	Subset for alpha = 0.05			
Cr^{VI} / kg soil	1	2	3	4
5 (20mg)	5.2705			
3 (5mg)	6.2918	6.2918		
4 (10mg)	6.4807	6.4807		
6 (30mg)		7.8835		
7 (50mg)			10.3360	
2 (1mg)				12.7216
1 (Control)				13.2682
8 (70mg)				14.9694
Sig.	.295	.173	1.000	.060

Means for groups in homogeneous subsets are displayed.

Generally, each plant type has an inherent normal root/shoot ratio under healthy environmental conditions and it clearly indicates normal balanced growth of the plant, but any decrease in the root/shoot ratio from the normal level indicates the plant is encountering or has encountered some kind of stresses. The present study inferred a progressive reduction in the root to shoot ratio from the normal level of 15.05 root/shoot fresh weight ratio as well as from 13.27 root/shoot dry weight ratio in *A. dubius* plants treated with Cr^{VI} up to a concentration of 20mg/kg soil. This observation in the study can

be attributed to the sequestration of toxic chromium in vacuoles of root cells which in turn may cause disturbed cell division and elongation of root cells followed by decline in the root growth and impairment of the root penetration (Zou *et al*., 2006; Oliveira, 2012; Kumar & Maiti, 2013). This is because, the root system is the part of the plant which is first affected by the Cr^{VI} toxicity in the soil as it is the part which is in direct contact with the toxic soil environment. However, the study noticed that, further enhancement in the concentration of Cr^{VI} up to 70mg/kg soil caused a progressive increase in root/shoot ratio from the reduced root/shoot ratios during initial levels of Cr^{VI} stress. This might be due to enhanced reduction in shoot growth which is as a result of severe Cr^{VI} toxicity induced root injury. The root injury may cause considerable reduction in absorption as well as translocation of root-zone resources such as water and nutrients into the shoot system for their utilization into biomass yield by making use of above ground resources such CO_2 and sunlight.

Dry weight/ Fresh weight ratio (DW/FW ratio)

The lowest Cr^{VI} treatment of 1mg recorded highest dry weight/fresh weight ratio for shoot, root and total plant in *Amaranthus dubius.* This ratio tended to exhibit a progressive decrease up to a concentration of 30mg Cr^{VI} treatment with respect to shoot and total plant while it is only up to 20mg Cr^{VI} treatment with respect to root component. However, further increase in the concentration of Cr^{VI} has caused a progressive increasing trend up to 70mg Cr^{VI} treatment, although any of the values did not exceed over control (table 2.19 and figure 2.5).

The highest DW/FW ratio of shoot, root and total plant of *A. dubius* recorded by 1mg Cr^{VI} treatment in the study did not differ significantly over control but differed significantly higher over all other treated plants. The lowest ratio of shoot as well as total plant induced by 30mg Cr^{VI} treatment is insignificantly lower over 50mg & 70mg Cr^{VI} treatments with respect to shoot component while it is insignificantly lower over 50mg Cr^{VI} treatment alone with respect to total plant. The lowest DW/FW ratio of root component induced by 20mg Cr^{VI} treatment is insignificantly lower over 10mg Cr^{VI}

treatment alone. The DW/FW ratio obtained with rest of the treatments in all the cases significantly differed over lowest ratios (table 2.20, 2.21 & 2.22).

Table 2.19: Effects of different concentrations of Cr^{VI} stress on **dry weight/fresh weight ratio** of shoot, root and total plant in *Amaranthus dubius*

Treatment Code Cr^{VI} / kg soil	Dry weight / Fresh weight ratio (DW/FW Ratio)					
	Shoot	SD±	Root	SD±	Total plant	SD±
1 (Control)	9.76	0.14	8.61	0.20	9.61	0.11
2 (1mg)	9.82	0.15	8.56	0.12	9.66	0.15
3 (5mg)	8.67	0.28	6.74	0.11	8.52	0.28
4 (10mg)	6.72	0.18	5.47	0.22	6.62	0.15
5 (20mg)	6.58	0.40	5.21	0.19	6.49	0.37
6 (30mg)	5.61	0.34	6.00	0.54	5.63	0.36
7 (50mg)	5.98	0.23	7.36	0.41	6.04	0.19
8 (70mg)	6.06	0.29	7.45	0.30	6.18	0.25

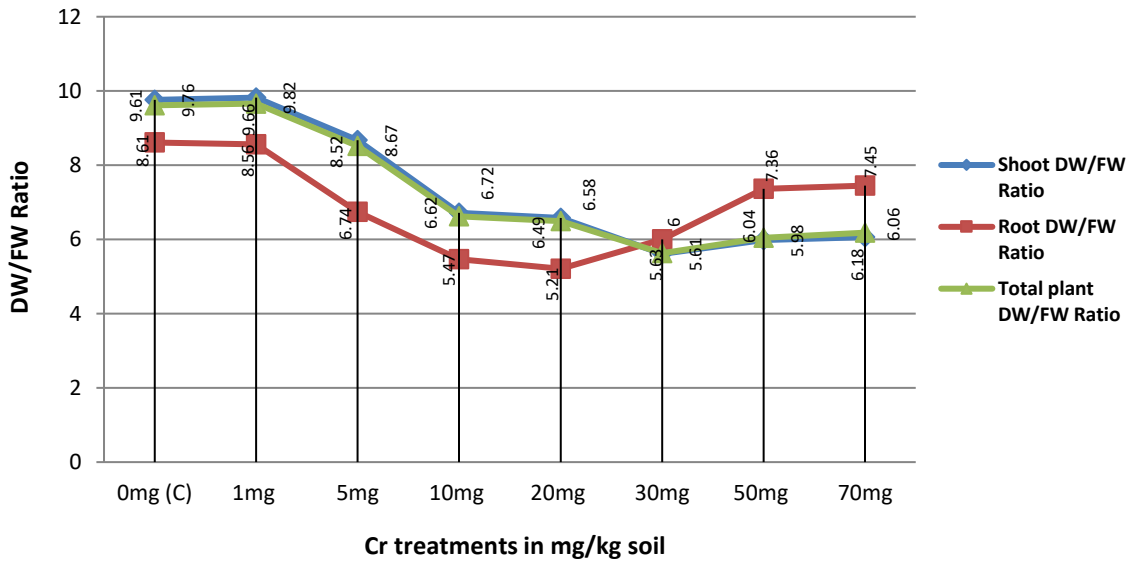


Figure 2.5: Effects of different concentrations of Cr^{VI} on Dry weight/Fresh weight ratio in *A. dubius*

Table 2.20: Duncan analysis of **shoot dry weight/fresh weight ratio** variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
6 (30mg)	5.6100			
7 (50mg)	5.9800			
8 (70mg)	6.0600			
5 (20mg)		6.5800		
4 (10mg)		6.7200		
3 (5mg)			8.6700	
1 (Control)				9.7600
2 (1mg)				9.8233
Sig.	.066	.528	1.000	.774

Means for groups in homogeneous subsets are displayed.

Table 2.21: Duncan analysis of r**oot dry weight/fresh weight ratio** variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
5 (20mg)	5.2067				
4 (10mg)	5.4700				
6 (30mg)		6.0000			
3 (5mg)			6.7400		
7 (50mg)				7.3600	
8 (70mg)				7.4500	
2 (1mg)					8.5600
1 (Control)					8.6100
Sig.	.292	1.000	1.000	.714	.839

Means for groups in homogeneous subsets are displayed.

Table 2.22: Duncan analysis of **total plant dry weight/fresh weight ratio** variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
6 (30mg)	5.6340				
7 (50mg)	6.0413	6.0413			
8 (70mg)		6.1780	6.1780		
5 (20mg)			6.4940		
4 (10mg)			6.6243		
3 (5mg)				8.5233	
1 (Control)					9.6103
2 (1mg)					9.6620
Sig.	.062	.510	.052	1.000	.802

Means for groups in homogeneous subsets are displayed.

The decreasing trend in the DW/FW ratio of shoot, root and total plant under comparatively lower Cr^{VI} toxicity observed in *A. dubius* can be attributed to Cr^{VI} stress induced decrement in the water content of the plant body due to reduced uptake and mobilization of water by the poorly developed and suberized root system. However, the severe Cr^{VI} toxicity at higher concentration treatment like 50mg/kg soil and above may cause considerable damage to root cells and this might have severely affected the water absorption process by the poorly developed root system. This in turn might have caused considerable reduction in the difference in fresh and dry weight of the plant body and this may be the reason for an increasing trend in the DW/FW ratio at higher Cr^{VI} toxicity levels.

Average leaf number/plant, Average area/leaf and Total leaf area/per plant

The effect of different concentrations of Cr^{VI} stress on total number of leaf/plants, average area/leaf and total leaf area/plant in *Amaranthus dubius* is shown in table 2.23. All the Cr^{VI} treatments have caused a reduction in all the leaf parameters over control, with an exception in the case of average area/leaf with respect to 1mg Cr^{VI} treatment which caused an insignificant increase. The analysis of the data generally revealed a progressive reduction in all the leaf parameters along with the increase in concentration

of Cr^{VI} treatments, with the highest values being recorded in 1mg Cr^{VI} treatment and the lowest values in 70mg Cr^{VI} treatment (figure 2.6, 2.7 & 2.8).

Table 2.23: Effects of different concentrations of Cr^{VI} stress on total number of leaf/plants, average area/leaf and total leaf area/plant in *Amaranthus dubius*

Treatment Cr^{VI} / kg soil	Leaf parameters/Plant					
	Total no of leaf	SD±	Average area/Leaf	SD±	Total leaf area/plant	SD±
0mg (Control)	12.75	0.5	30.84	7.7	362.12	94.7
1mg	12.25	1.0	31.21	6.6	345.74	67.1
5mg	11.50	1.3	9.90	1.1	123.57	11.1
10mg	8.75	1.5	12.25	2.2	90.05	15.5
20mg	7.75	1.0	9.41	0.4	72.29	10.4
30mg	7.00	1.4	4.72	0.1	31.08	6.6
50mg	4.50	0.6	2.87	0.2	13.82	1.6
70mg	3.75	1.0	2.31	0.1	10.57	4.8

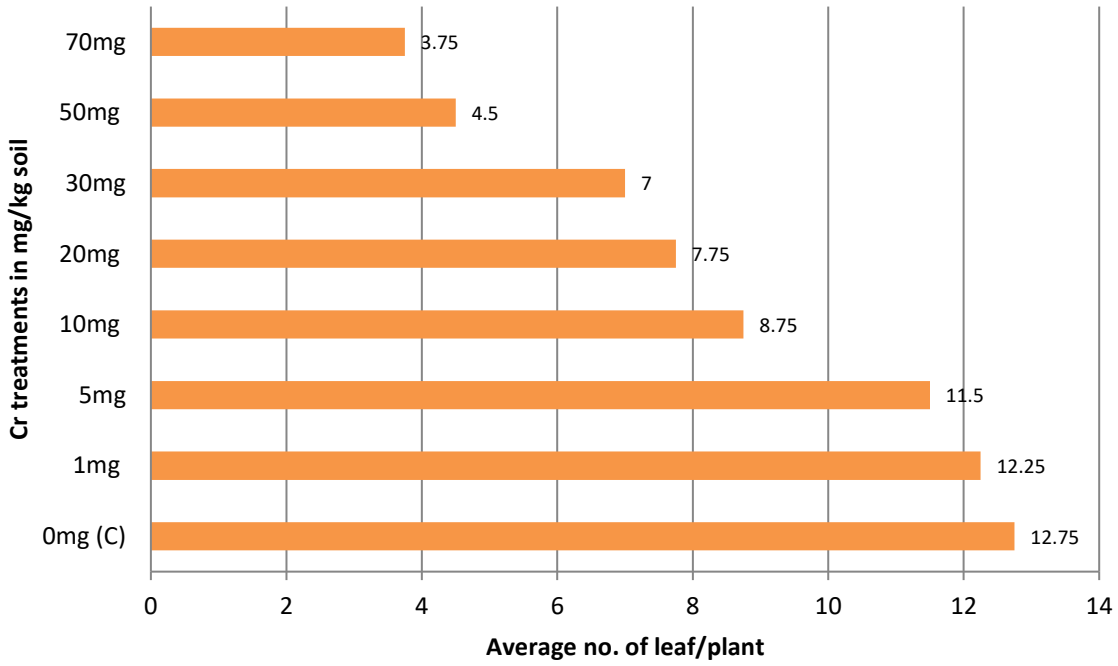


Figure 2.6: Effects of different concentrations of Cr^{VI} on total number of leaves in *A. dubius*

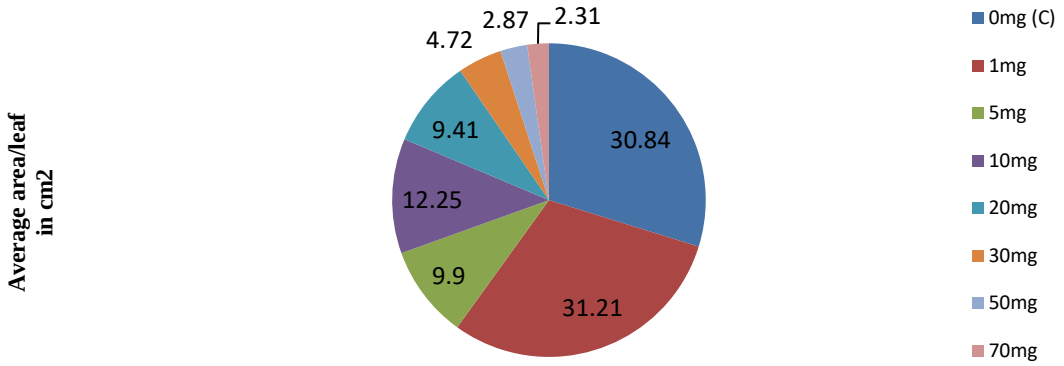


Figure 2.7: Effects of different concentrations of Cr^{VI} stress on average area/leaf in *A. dubius*

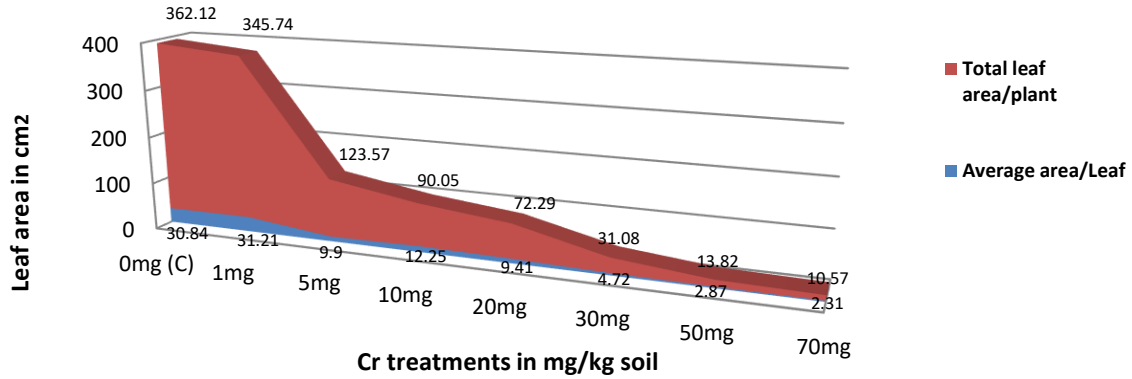


Figure 2.8: Effects of different concentrations of Cr^{VI} on leaf area in *Amaranthus dubius*

The average leaf number of 12.25 and 11.5 obtained in 1mg and 5mg Cr^{VI} treatment respectively did not differ significantly over the average leaf number of 12.75 obtained in control while the leaf number obtained with all other treatments differed significantly lower. Similarly, the average area/leaf of 31.21 cm^2 and average leaf area/plant of 345.74 cm^2 obtained in 1mg Cr^{VI} treatment is insignificant over control while the average values obtained with all other treatments differed significantly lower over control values of 30.84cm^2 and 362.12 cm^2 respectively for average area/leaf and average leaf area/plant (table 2.24, 2.25 & 2.26).

Table 2.24: Duncan analysis on the **total leaf number/plant** in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	3.75			
7 (50mg)	4.50			
6 (30mg)		7.00		
5 (20mg)		7.75	7.75	
4 (10mg)			8.75	
3 (5mg)				11.50
2 (1mg)				12.25
1 (Control)				12.75
Sig.	.334	.334	.201	.132

Means for groups in homogeneous subsets are displayed.

Table 2.25: Duncan analysis on the **average area/leaf** in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	2.31			
7 (50mg)	2.87			
6 (30mg)	4.72	4.72		
5 (20mg)		9.41	9.41	
3 (5mg)		9.90	9.90	
4 (10mg)			12.25	
1 (Control)				30.84
2 (1mg)				31.21
Sig.	.393	.071	.314	.888

Means for groups in homogeneous subsets are displayed.

Table 2.26: Duncan analysis on the **total leaf area/plant** in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	10.57			
7 (50mg)	13.82			
6 (30mg)	31.08	31.08		
5 (20mg)	72.29	72.29	72.29	
4 (10mg)		90.05	90.05	
3 (5mg)			123.57	
2 (1mg)				345.74
1 (Control)				362.12
Sig.	.066	.070	.113	.585

Means for groups in homogeneous subsets are displayed.

The leaf morphological as well as physiological characteristics of plants are generally highly sensitive to heavy metal stress and the major visible impacts that we can visualize as morphological parameters include decrease in leaf number and size and hence reduction in total leaf area of the plant. The present study inferences are in agreement with above generalizations and further revealed the reductions in leaf parameters are more or less progressive and positively correlated with increase in Cr^{VI} stress. The reductions in the leaf number and leaf area of Cr^{VI} treated plants over the control could be due to the inhibitory effect of chromium toxicity on two major physiological processes such as cell division and expansion (Hall, 2002; Poschenrieder & Barceló, 2004; Nagajyoti *et al.*, 2010; Marschner, 2011). This can be further attributed to adverse effects of Cr^{VI} toxicity on hydraulic conductance and water potential of plant tissues and restrictions in the uptake of mineral nutrients like nitrate, potassium etc (Hall, 2002; Seregin & Ivanov, 2001; Poschenrieder & Barceló, 2004; Nagajyoti *et al*., 2010).

Tolerance index (TI)

Table 2.27: Tolerance index (TI) of *A. dubius* against different concentrations of Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Tolerance index (TI) in % based on					
	Root length	SD±	Fresh biomass	SD±	Dry biomass	SD±
0mg (Control)	100.00	0.0	100.00	0.0	100.00	0.0
1mg	118.23	16.0	88.92	2.7	89.39	2.4
5mg	32.07	3.4	17.64	0.28	15.65	0.5
10mg	26.93	5.4	12.18	3.5	8.45	2.3
20mg	27.34	2.3	12.07	1.6	8.17	1.1
30mg	23.00	7.7	9.39	0.8	5.50	0.6
50mg	17.94	3.6	6.07	0.5	3.82	0.3
70mg	13.90	3.7	2.89	0.5	1.86	0.4

The tolerance index of *Amaranthus dubius* against different concentrations of Cr^{VI} stress is depicted in the table 2.27. With an exception of 1mg Cr^{VI} treatment with respect to root length, all the Cr^{VI} treatments have been found to have reduced tolerance index when compared to control in terms of root length, fresh biomass and dry biomass. 1mg Cr^{VI} treatment caused the lowest reduction in tolerance index of 11.08% in terms of fresh biomass and 10.61% in terms of dry biomass accumulation, which differed significantly lower over control and significantly higher over all other concentration treatments (table 2.29 & 2.30). With respect to root length, the lowest reduction is recorded in 5mg Cr^{VI} treatment which did not differ significantly over 10mg, 20mg and 30mg Cr^{VI} treatments while 1mg Cr^{VI} treatment caused a significant enhancement of 18.23% tolerance index over control (table 2.28). The results generally revealed that the increase in Cr^{VI} stress induced a progressive reduction in tolerance index and the highest reduction of 86.1%, 97.11% and 98.14% is recorded in 70mg Cr^{VI} treatment respectively for root length, fresh biomass and dry biomass (figure 2.9). The decrease in tolerance index of *A. dubius* plants due to Cr^{VI} treatments clearly indicate the toxic effect. Further, the progressive reduction in tolerance index inferred in the study along with the increase in concentration of Cr^{VI} treatments revealed dose dependent toxicity level on various growth parameters.

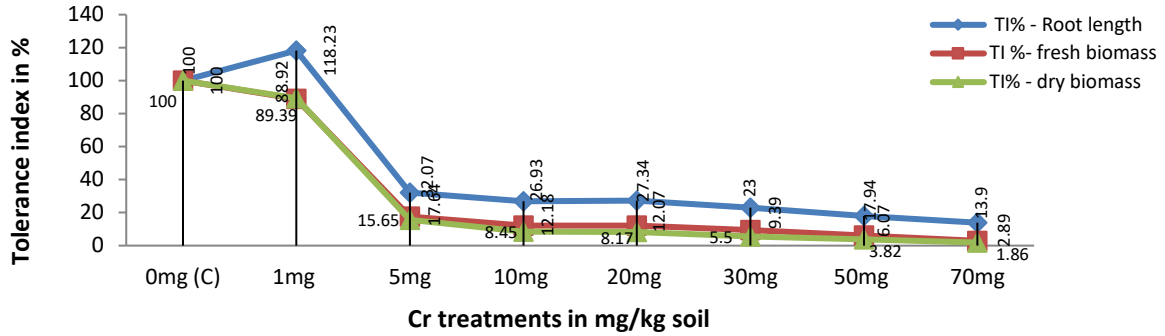


Figure 2.9: Tolerance index (TI) of *A. dubius* against different levels of Cr^{VI} stress

Table 2.28: Duncan analysis of TI based on the root length in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	13.90				
7 (50mg)	17.94	17.94			
6 (30mg)	23.00	23.00	23.00		
4 (10mg)		26.93	26.93		
5 (20mg)		27.34	27.34		
3 (5mg)			32.07		
1 (Control)				100.00	
2 (1mg)					118.23
Sig.	.093	.092	.104	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 2.29: Duncan analysis of TI based on the fresh biomass in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
8 (70mg)	2.89						
7 (50mg)		6.07					
6 (30mg)			9.39				
5 (20mg)				12.07			
4 (10mg)				12.18			
3 (5mg)					17.64		
2 (1mg)						88.92	
1 (Control)							100.00
Sig.	1.000	1.000	1.000	.930	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 2.30: Duncan analysis of TI based on the dry biomass in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
8 (70mg)	1.86					
7 (50mg)		3.82				
6 (30mg)		5.50				
5 (20mg)			8.17			
4 (10mg)			8.45			
3 (5mg)				15.65		
2 (1mg)					89.39	
1 (Control)						100.00
Sig.	1.000	.076	.759	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Inhibition of Plant Growth (IPG in %)

The details of inhibition of plant growth (IPG in %) due to different levels of Cr^{VI} stress is depicted in the table 2.31. The lowest Cr^{VI} stress treatment of 1mg concentration has caused an increase in plant length over control in *Amaranthus dubius* while the rest of the Cr^{V} treatments caused a reduction. In terms of plant length, the lowest IPG of 50.93% obtained in 5mg Cr^{VI} stress treatment while in terms of fresh and dry biomass accumulation, the lowest IPG of 11.11% and 10.61% respectively obtained with 1mg Cr^{VI} stress treatment. The data revealed that further increase in Cr^{VI} stress is causing a progressive enhancement in IPG and the highest IPG of 78.83%, 97.07% and 98.13% respectively in terms of plant length, fresh and dry biomass is obtained in the highest Cr^{VI} stress of 70mg concentration (table 2.31 & figure 2.10).

Table 2.31: Inhibition of plant growth (IPG in %) in *Amaranthus dubius* against different levels of Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Inhibition of plant growth (IPG in %) based on					
	Plant length	SD±	Fresh biomass	SD±	Dry biomass	SD±
0mg (Control)	0.00	0.0	0.00	0.0	0.00	0.0
1mg	-4.47	1.8	11.11	3.3	10.61	2.9
5mg	50.93	7.7	82.37	0.4	84.36	0.6
10mg	60.33	2.4	87.80	4.2	91.61	2.8
20mg	61.77	5.3	87.97	1.9	91.86	1.4
30mg	70.20	0.5	90.63	1.0	94.50	0.7
50mg	74.07	1.8	93.93	0.7	96.94	1.6
70mg	78.83	0.6	97.07	0.6	98.13	0.5

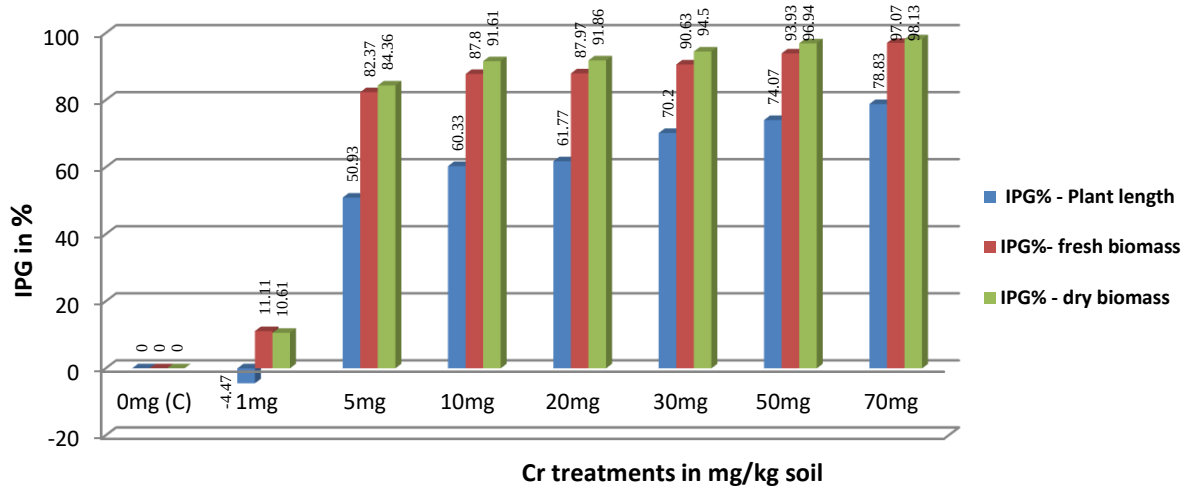


Figure 2.10: Inhibition of plant growth (IPG in %) in *A. dubius* against different levels of Cr^{VI} stress

The lowest IPG values obtained at1mg Cr^{VI} stress in terms of both fresh and dry biomass are significantly lower compared to the IPG values obtained with all other Cr^{VI} stress (table 2.33 & 2.34), while the lowest IPG values obtained in terms of plant length at 5mg concentration treatment differed significantly higher over 1mg Cr^{VI} stress whereas it differed significantly lower over rest of the Cr^{VI} stress treatments (table 2.32). The highest IPG value obtained at 70mg Cr^{VI} stress in all the cases and it is found only insignificantly higher over the values obtained with 50mg Cr^{VI} stress treatment while the differences over the rest of the treatments are significantly higher.

Table 2.32: Duncan analysis of IPG (%) in terms of plant length in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
2 (1mg)	-4.47				
1 (Control)	0.00				
3 (5mg)		50.93			
4 (10mg)			60.33		
5 (20mg)			61.77		
6 (30mg)				70.20	
7 (50mg)				74.07	74.07
8 (70mg)					78.83
Sig.	.141	1.000	.626	.199	.118

Means for groups in homogeneous subsets are displayed.

Table 2.33: Duncan analysis of IPG (%) in terms of fresh biomass in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
1 (Control)	0.00					
2 (1mg)		11.11				
3 (5mg)			82.37			
4 (10mg)				87.80		
5 (20mg)				87.97		
6 (30mg)				90.63	90.63	
7 (50mg)					93.93	93.93
8 (70mg)						97.07
Sig.	1.000	1.000	1.000	.129	.068	.081

Means for groups in homogeneous subsets are displayed.

Table 2.34: Duncan analysis of IPG (%) in terms of **dry biomass** in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
1 (Control)	0.00					
2 (1mg)		10.61				
3 (5mg)			84.36			
4 (10mg)				91.61		
5 (20mg)				91.86		
6 (30mg)				94.50	94.50	
7 (50mg)					96.94	96.94
8 (70mg)						98.13
Sig.	1.000	1.000	1.000	.059	.092	.393

Means for groups in homogeneous subsets are displayed.

The inferences obtained from the analysis of different morphological growth parameters in the study clearly indicated the inhibition of growth in *A. dubius* plants treated with hexavalent chromium (Cr^{VI}). This could be due to toxicity induced damage and poor development of root system. Root injury and reduced root growth can restrict the absorption of water and various essential elements in the soil root zone and their mobilization into different regions of plants to carryout various physiological processes to ultimately yield biomass. The present study on the morphological growth parameters of *A. dubius* in response to Cr^{VI} stress revealed the existence of a positive correlation between the inhibition of growth and the intensity of Cr^{VI} stress. This can be attributed to the differences in Cr^{VI} toxicity induced extension of cell cycle and related inhibition in cell division and enlargement, which in turn may be due to the differences in the intensity of Cr^{VI} stress induced alteration or imbalances in physiological and biochemical processes. This may include disturbances in biosynthesis of photosynthetic pigments, reduction in stomatal conductance and gas exchange activities, disruption in electron transport system etc which are undesirably affecting the rate of photosynthesis, ATP production and energy level of cells.

CONCLUSION

The study revealed that hexavalent chromium (Cr^{VI}) stress has significantly deleterious effects on the morphological growth parameters of *Amaranthus dubius*. With certain exceptions in the initial level concentration of 1mg Cr^{VI} stress treatment, the study revealed that all the concentration treatments have induced an inhibitory effect on all the morphological parameters that would contribute to the productivity of the crop. The different responses in terms of growth in length and biomass accumulation of root, shoot and total plant and similarly the total number of leaves and total leaf area per plant and average area per leaf, generally exhibited a progressive reduction along with the increase in the concentration of the metal Cr^{VI}. The assessment of tolerance index and percentage of growth inhibition in *A. dubius* in response to Cr^{VI} stresses revealed a progressive increase over the inhibition of plant growth and a corresponding reduction in tolerance index (TI). This indicates dose dependency of Cr^{VI} toxicity on growth parameters. Therefore, it can be concluded that there exist of a positive correlation between the inhibition of growth and intensity of Cr^{VI} stress. The oxidative stress induced by Cr^{VI} toxicity caused reduction in root growth, root injury and impaired root penetration in *A. dubius* which have led to restricted absorption and translocation of water and mineral nutrients to the aerial parts of the plant causing disruption in carrying out normal growth and development to yield the biomass. The past research and related literatures on heavy metal toxicity hinds that the oxidative stress induced by excessive concentration of heavy metal in the growing environment can cause imbalance and alterations in the normal physiology and metabolism of plants. These disrupted processes may include disturbances in biosynthesis of photosynthetic pigments, stomatal conductance and related gas exchange activities, which can undesirably influence the net photosynthesis. Further, the metal toxicity is also known to cause chromosomal aberrations, disruption in structural organization and integrity of cell, disturbed cell division and enlargement. The Cr^{VI} toxicity induced disruption in all these processes additively might have contributed to the significant reduction in overall morphological growth and biomass parameters of

A. dubius. Therefore, it is highly relevant to evaluate these processes under Cr^{VI} stress to scientifically prove and confirm toxicity.

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CHAPTER-3

PHYSIOLOGICAL AND BIOCHEMICAL ALTERATIONS INDUCED UNDER HEXAVALENT CHROMIUM (Cr^{VI}) STRESS

INTRODUCTION

It has not been conclusively evident that Chromium has a significant role in the metabolism and physiology of plants. There has been a disagreement in the literature results in this aspect even though most of the studies stipulate the non-essentiality of Cr in plants (Hayat *et al.*, 2012), few studies observe that Cr in minor quantities produce a stimulating effect on productivity and growth of plants (Ghosh & Singh, 2005; Zayed & Terry, 2003). There have been more investigations carried out on the toxic effect of Cr on growth and development of plants. When available in lesser concentrations, Cr might induce some specific adaptive response in plant tissues enabling the plant to exhibit tolerance to this heavy metal without considerable ill effects. On the other hand, the defense and tolerance structure of the plant fail to cope up completely with the toxicity of Cr, when available in higher concentrations. When the metal stress is limited, the enzymatic and nonenzymatic defense systems are so regulated as to assist the redox balance thereby helping the repair of oxidative damage (Maiti *et al*., 2012; Olteanu *et al*., 2012). However, if the capability of the protective network is insufficient, there may be a significant increase in the modification of biomolecules causing oxidative stress with corresponding adverse effects on plants. In the general sense, it is understood that excess concentrations of Cr may induce many morpho-physiological growth processes in plants (UdDin *et al*., 2015; Kamran *et al*., 2017). The studies conducted earlier have reported that Cr hinders the gas exchange parameters comprising of evapotranspiration, stomatal conductance and CO_2 assimilation (Rodriguez *et al*., 2012). In addition to this, it is reported that water imbalance in plants influencing stomatal opening is caused by the toxicity of Cr leading to instability in gas exchange affairs (Barbosa *et al*., 2007). This may interrupt photosynthesis with regard to photophosphorylation, carbon assimilation, alterations in Rubisco activity and electron transport (Pandey & Sharma, 2003). Further, many enzymes are restricted at higher concentrations of Cr (Vazques *et al*., 1987) leading to decreased photosynthetic yield (Nagajyoti *et al*., 2010).

The metal ions exert a biochemical effect on plant cells which is equally diverse as its chemical characteristics and endurance to toxicity caused by heavy metals are species specific and variety specific for crop plants (Metwally *et al*., 2005). Variations in the protein constitution and content is identified as one of the oxidative strains caused by heavy metal toxicity (Pant & Tripathi, 2014). The biological activity of a protein is identified to be obstructed by heavy metals by changing the native configuration by way of binding on it (Hossain & Komatsu, 2013). Palma *et al*. (2002) identified that there is a reduction in the protein content as a result of higher degradation action of protein caused by increased protease activity and lipid peroxidation induction together with protein fragmentation. The repressive nature of nitrate reductase action may be the cause of reduction in protein matter in plants when subjected to heavy metal stress (Vajpayee *et al*., 2000). At the same time, some reports identify an increase in protein level content in plants that are exposed to excessive levels of heavy metals as a result of the improved production of stress linked proteins like HSPs, proteinases, proline and pathogenesis related proteins (Lee *et al*., 2010). Observations by Singh and Pandey (2011) is that under low concentrations of heavy metals, there is an increase in protein content in the leaves of *Pistia stratiotes* which got decreased at higher concentrations. There are reports of starch accumulation in plants subjected to metal stress. Tian *et al.* (2010) observed the accumulation of starch in leaves of Arabidopsis under heavy metal stress and enhanced starch content in Bean leaves under Pb and Ni stress has been reported by Nyitrai *et al*. (2004), while accumulation of starch granules in damaged root cells of citrus is reported by Zambrosi *et al*. (2013). All the above reports propose the defects of metal stress caused in phloem loading or translocation which might have disturbed the export or consumption of assimilates which ultimately led to starch accumulation. Costa and spitz (1997) observed that in certain plants, the carbohydrate and amino acid levels increased in shoots but not in roots at lower concentrations of heavy metals whereas at higher concentrations, all primary metabolites retarded. A decrease in total sugar content when subjected to heavy metal stress has been reported in many research works in different crop plants such as Gram (Tandon & Gupta, 2002), *Trigonella foenumgraecum* (Patel, 2008), rice (Yeh *et al*., 2006), sugar beet (Greger & Lindberg, 1986) and wheat (Singh *et*

al., 2007). However, there are exceptions observed in certain plants where an increase is noticed at low level concentration of heavy metals but found to decrease as the concentration further increased (Zhao *et al*., 2016; Deef, 2007). The hypothesis proposed by Greger *et al*. (1991) state that heavy metal stress can cause a considerable effect in reducing the transportation of carbohydrate and thereby lowering photosynthesis. There are earlier reports of increase in soluble carbohydrate concentration and decrease in starch concentration in the seedlings of *Pinus sylvestris* when there is a hike in the heavy metal concentration in the growing media which indicate an imbalance in the regulation of carbohydrate under conditions of heavy metal stress (Kim *et al*. 2003; Guangqiu *et al*., 2007). Over production of Reactive Oxygen Species (ROS) like hydroxyl radicals (OH^-), superoxide ($O2^-$), the paramagnetic singlet oxygen ($^1O2$), nitrogen oxide radical (NO), hydrogen peroxide (H_2O_2) molecules etc. is another customary and recurring effect of heavy metal stress (Valko *et al*., 2007; Pisoschi & Pop, 2015). It may be understood that the toxic effects of Cr and other heavy metals in plants might be a consequence of over production of these reactive oxygen species (ROS) eventually leading to disruption of the redox balance (Morina *et al*., 2010; Anjum *et al*., 2017; Sharma *et al*., 2019), causing drastic and acute imbalances in both physiological and biochemical growth process. Taking into account all the above observations, the present chapter analyzes the impact of different concentration levels of hexavalent chromium (Cr^{VI}) stress on various physiological and biochemical growth parameters of *Amaranthus dubius*. This study further aims to evaluate the changing trends in the physiological and biochemical parameters selected for the study and whether they are in agreement with the changes in the morphological growth parameters obtained against different concentrations of Cr^{VI} stress in the previous chapter.

REVIEW OF LITERATURE

There are reports that Chromium (Cr) toxicity retards plant growth and obstructs the essential metabolic processes (Shanker *et al*., 2009). The unfavourable effects of Cr toxicity are the reduction in plant growth by bringing about ultrastructural modifications of the cell membrane and chloroplast, persuading damaging root cells, reducing pigment content and mineral nutrition, chlorosis in the leaves, affecting transpiration and nitrogen assimilation, disturbing water relations and by altering different enzymatic activities (Reale *et al*., 2016; Ali *et al*., 2015; Anjum *et al*., 2017). The harmful effects of Chromium (Cr) also include the repercussions on the physiological processes like photosynthesis, water relations and mineral nutrition (Nagajyoti *et al*., 2010; Shanker *et al*., 2005), which in turn can give rise to morphological changes (Daud *et al*., 2014; Rodriguez *et al*., 2012; Singh *et al*., 2013).

The presence of excess amount of heavy metal in the soil has an adverse effect on the seed germination, has the ability to alter the level of biomolecules in the cells and can interfere with the functioning of many vital enzymes associated with normal physiology, metabolism and growth (Zhang *et al*., 2009; Rahoui *et al*., 2010). There are observations that heavy metals hinder the various physiological activities as a constituent of many enzymes mainly that take part in electron flow, catalyze redox reactions in mitochondria and chloroplasts (Hansch & Mendel, 2009). Metals are identified to suppress the uptake of O_2 causing an inhibitory effect on the standard physiological exercises and thus growth (Sharma & Sharma, 1996). The negative effects taking place in leaves due to Cr exposure are suppression of chlorophyll synthesis, inhibition of photosynthetic electron transport, disruption of chloroplast ultrastructure, and release of magnesium ions from the molecule of chlorophyll (Panda & Choudhury, 2005; Shah *et al.*, 2010; Rai *et al*., 2004).

Chlorophyll biosynthesis is another activity that is inhibited by excess chromium (Cr) and it is reported in terrestrial plants (Vajpayee *et al*., 2000). Reduction in the intake of Mg and N which are the major components of chlorophyll molecule may be the cause of reduction in chlorophyll pigments, as a result of Cr stress (Sela *et al*., 1989). Impairment in the functioning of enzymes like δ-aminolaevulinic acid dehydratase (ALAD or

porphobilinogen synthase) and protochloro- phyllide reductase, which are essential for chlorophyll biosynthesis, could be the reason for reduced chlorophyll content in exposed to Cr stress (Ganesh *et al*., 2008).The suppressed ALAD activity leading to inadequate PBG (porphobilinogen),which is necessary for chlorophyll synthesis, may be attributed to curtailment in chlorophyll pigments when plants are subjected to Cr toxicity (Prasad & Prasad 1987).

Invariably, all elements, both directly and indirectly, influence the photosynthetic apparatus causing retardation in photosynthesis when plants are under metal stress (Sytar *et al*., 2013). Chromium toxicity induced modifications include less amounts of grannae, alterations in the ultrastructure of chloroplasts, a poorly developed lamellar structure with widely spaced thylakoids which all can adversely affect photosynthetic pigments and photosynthesis (Bishehkolaei *et al*., 2011). In *Oryza sativa* L. cultured with 50, 100, 150, 200, 300, 400 and 500 mg kg^{-1} of Cr^{6+}, it has been identified that there is a reduction in photosynthetic rate, transpiration and stomatal conductance when compared to control plants which is indicative of a diminishing rate of gas exchange parameters and chlorophyll a, b and carotenoids, with increment in Cr^{6+} concentrations (Ahmad *et al*., 2011). Reports available indicate that Cr has a negative effect on the assimilation of CO_2 and inhibits net photosynthetic rate (Vernay *et al*., 2007; Liu *et al*., 2008; Subrahmanyam, 2008). This may be on account of the alterations in the functioning of carbon fixation enzymes and electron transport chain (Larcher, 1995). Van and Clijsters (1983) inferred that the disorganization of the chloroplast ultrastructure may be reason for reduced photosynthetic yield in the presence of Cr^{6+} and Shanker *et al*. (2005) observed that the blockage of electron transport processes is attributed to the deviation of electrons from the PSI electron donor side. It is also identified that a portion of the electrons produced by photochemical process might not be utilized for carbon sequestration as exhibited by low photosynthetic output in plants subjected to Cr stress, but on the other hand, they may be taken up for reduction in oxygen molecules which may describe the oxidative stress due to Cr (Shanker *et al*., 2005). Plant biomass reduction on account of inhibited CO_2 absorption and photosynthesis caused by hexavalent chromium has been reported by Singh *et al*. in 2013. The activities such as respiration, symbiotic nitrogen

fixation and photosynthesis are found to decrease significantly in pea plants when Cr in the form of potassium dichromate is added (Pandey *et al*., 2013). The study on chloroplasts isolated from *Beta vulgaris* L. which are subjected to Cr^{6+} stress shows a considerable obstruction in electron transport activity in PSI and PSII (Pandey *et al*., 2013). The functions of the photosystem I (PSI) and the photosystem II (PSII), which are found in the thylakoid membranes, are adversely affected by metals and PSII is identified to be more vulnerable to the toxicity of metals compared to PSI (Sytar *et al*., 2013). According to Appenroth *et al*. (2001), reduction in the number of plastoquinone B bonding sites may be the reason for increase in the number of inactive PSII units in the presence of Cr.

Plants under Cr^{VI} stress have reduced transpiration rate which is attributed to the negative effect on stomatal resistance in leaves. The efficiency of water flow is hampered under excessive heavy metal influence by bringing down the transpiration rate caused by changes in the stomal resistance in leaves (Barceló & Poschenrieder, 1990). When transpiration (E) increases, the leaf water potential and the abscisic acid (ABA) in the leaves decreases activating a response that give rise to the feedback hypothesis which states that stomatal conductance decreases as VPD increases (Bunce, 1997). Plants that are exposed to heavy metals may prompt to increase many signaling compounds like abscisic acid (ABA), which interfere with the signal pathways under conditions of water stress resulting in speedy induction of stomata closure, causing reduced water loss in leaves (Rucińska, 2016).Tomato plants, when subjected to different levels of Cr stress express reduction in stomatal conductance, internal carbon and rate of photosynthesis and these observations shows that the toxic effects of Cr^{6+} can bring about deficiencies in PSII directly or indirectly (Henriques, 2010).

The defense mechanism exhibited by plants to fight the stress caused by the toxicity and intolerance due to HMs include metal sequestration and compartmentalization, biosynthesis or accumulation of osmolytes and osmoprotectants, metal binding to cell wall, avoidance, metal ions trafficking etc. (Patra *et al*., 2004; Memon *et al*., 2001; John, 2009; Dalvi & Bhalerao, 2013). In a situation where the above strategies fail and the

plants get devastated with heavy metal toxicity, the initiation of antioxidant defense technique is sought after (Manara, 2012). When all the above defense mechanisms collapse, equilibrium of cellular redox systems in plants gets upset causing an increased induction of ROS (Mourato *et al*., 2012). To reduce the damaging effects of free radicals, the plant cells evolve many defense techniques which comprises of enzymatic antioxidants such as glutathione reductase(GR), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and superoxide dismutase (SOD) and nonenzymatic antioxidants via ascorbate (AsA), glutathione (GSH), carotenoids, alkaloids, tocopherols, proline, and phenolic compounds (flavonoids, tannins, and lignin), all of them performing the role of scavengers of free radicals (Sharma, 2012; Michalak, 2006). Plants may be subjected to several radical physiological changes as a result of the imbalances in the production and scavenging of ROS which is termed as an oxidative burst (Sharma *et al*., 2019; Anjum *et al*., 2015; Morina *et al*., 2010). When certain heavy metals like copper (Cu), nickel (Ni), cadmium (Cd), chromium (Cr) and arsenic (As) are present in excessive quantity, they show a tendency to generate ROS (Körpe & Aras, 2011).

The chromium metal (Cr) has the capacity to block the electron transport in plant mitochondria by way of replacing iron and copper ions in prosthetic groups of several carrier proteins (Singh *et al*., 2013; Dixit *et al*., 2002). Cr takes the place of central Mg ion in the chlorophyll molecule leading to impaired light reception and thereby collapse of photosynthetic activity (Küpper *et al*., 2002; Prasad & Strzałka, 1999). Dhir *et al*. (2009) identified that when *Salvinia natans* was exposed to waste water with abundance of Cr there was a considerable reduction in ribulose bisphosphate carboxylase oxygenase (RuBisCO) activity and proposed that this may be due to Mg^{2+} substitution by subunits metal ions in the active site of RuBisCo. As a result of this, there may be a depletion in the chlorophyll content as observed in other studies (Vajpayee *et al*., 2000). Dixit *et al*. (2002), observed that due to the changes in the redox reactions of Fe and Cu carriers, C^{6+} gets transferred through cytochrome mitochondria permitting it to be bonded to the heme group of cytochromes by interfering with transport electrons. Acute inhibition in the activity of enzymes like cytochrome a3 and Complex IV of cytochrome oxidase is noted by the binding of Cr^{6+} with them (Dixit *et al*., 2002).

Due to the intrusion of heavy metals with the metabolic and biochemical plant processes, several negative effects are reported which causes damage to internal and external root structures, decrease chlorophyll content, decrease root hydraulic water conductivity, retardation in root growth, interference with enzymatic activities related to photosynthesis, decrease in stomatal opening and conductance, suberization and interference with nutrient absorption and translocation leading to nutrient imbalance (Benavides *et al*., 2005; Zhang *et al*., 2010). Low water potential compared to nutrient uptake and modifications in the functioning of important enzymes of different metabolic pathways occurring under metal stress may cause retardation in plant growth pathways (Arduini *et al*., 1996) and disrupted arrangement of microtubule in meristematic cells (Eun *et al*., 2000). Accumulation of excess quantity of heavy metals in plants may cause modifications in the key protein structure or replace the essential elements which is displayed as inactivation of photosystems, chlorosis, browning of roots, growth impairment together with other effects, all inducing toxicity (Gorhe & Paszkowski, 2006; Tak *et al*., 2013).

Remarkable alterations are noticed in the biochemistry of photosynthesis when plants are subjected to heavy metal stress. Heavy metals cause an impact on the enzymes like pyruvate phosphoenol dikinase that catalyses photosynthesis (Doubnerova & Ryslava, 2011), PEPC enzyme that catalyze the reaction of bicarbonate and phosphoenol pyruvate (PEP) to produce 4 C acid compound oxaloacetic acid (OAA) which require divalent metal ions such as Mg^{+2} and Mn^{+2} for activation. Along with these ions, cofactors like Fe and Zn ions also get replaced by heavy metals resulting in inhibiting the enzyme activity leading to reduced CO_2 supply at rubisco site ultimately diminishing the net productivity. The competition between heavy metals like Cr, Cd etc. and essential metal ions like Fe, Mg, Zn, and Mn etc. can suppress the mineral nutrition (Solti *et al*., 2011).

Research works indicate that heavy metal toxicity can induce changes in metabolism and alter the biochemical parameters resulting in lesser growth rate and biomass matter of plants (Soni & Bhuva, 2015; Soni & Korgaokar, 2016). The basic target group of HMs is proteins. The HMs form an intricate structure with the active side chain groups of

proteins or take the place of inevitable ions from metallo proteins causing impairment in the physiological activities (Hossain & Komatsu, 2013; Tamás *et al*., 2014). Together with this, HMs also obstruct the native authentication of proteins by hampering the folding activity of the developing or non-native proteins that is evident from the deficiency of quantity of the attacked proteins and in the emergence of proteotoxic aggregates (Bierkens, 2000; Tamás *et al*., 2014). Protein synthesis may be restricted by heavy metals giving rise to decreased protein content as per Samantaray (2002). As the heavy metal concentration increases, total protein content is found to decrease, in comparison to control, in *Solanum lycopersicum* (Gulru & Cuneyt, 2019).

Chromium is a transition metal which engage in the process of cellular redox, especially in the production of Reactive Oxygen Species (ROS) and it is determined that ROS can associate with practically all cellular components such as lipids, carbohydrates, proteins, nucleic acids etc. causing oxidative damage (Panda & Choudhury, 2005a; Trinh *et al*., 2014). Chromium metal can cause degradation of proteins in plants leading to inhibition in the functioning of nitrate reductase (NR) (Solomonson & Barber, 1990; Panda & Choudhury, 2005). Rai *et al*. (1992) has documented the correlation between NR function and proteins in plants. Cr metal can alter the enzymatic functioning of proteins by its interactions either at the catalytic site or elsewhere, thereby deactivating the effective sites of enzymes by binding with distinct functional groups of proteins (Gupta *et al*., 2009; Gupta *et al*., 2010). In addition to this, displacement of important cations from the distinct enzyme binding sites occurs which causes disturbance in the equilibrium of ROS in cells, subsequently leading to generation of ROS in exorbitant amount (Shahzad *et al*., 2016).

The physiological changes taking place in plants that are subjected to heavy metal stress can remarkably reduce the synthesis of protein (Chaffei *et al*., 2004). Excessive concentration of metals may restrict protein metabolism by amending the physiological activities and synthetic functions (Sandalio *et al*., 2001). The decomposition of protein components is also affected by the toxicity of heavy metals in enhanced concentrations (Wu *et al*., 2014). Alteration in pigment-lipoprotein complex cumulation in photosystems

I causing inhibition of protein synthesis is noticed under higher concentrations of metals (Wang *et al*., 2009) along with adverse effect on ribulose-1,5-bisphosphate carboxylase/oxygenase enzymes (Krantev *et al*., 2008).

The carbohydrate contents in vegetables are strongly affected by heavy metals. Carbohydrate synthesis may be inhibited by interrupting the photosynthetic electron transport chain together with ROS production as a result of excess accumulation of elements with toxicity (Sandalio *et al*., 2001). Heavy metals are capable of inactivating and impairing some enzymes involved in the synthesis of carbohydrates thereby affecting carbohydrate metabolism leading to changes in their contents (Gawęda, 2007; Nagor & Vyas, 1997). Significant alteration in plant physiology causing reduced carbohydrate metabolism is another drawback of high heavy metal concentration (Chaffei *et al*., 2004).

MATERIALS AND METHODS

Analysis of Physiological parameters

Fully expanded leaves from top towards the middle of *Amaranthus dubius* seedling are selected for the analysis and measurements of various physiological parameters.

Chlorophyll parameters (Arnon, 1949)

Principle: Chlorophyll is extracted in 80% acetone and the absorption at 663nm and 645nm are read in a Spectrophotometer. From the absorption coefficients the chlorophyll is estimated.

Procedure: Take 1g of leaf sample and grind it thoroughly with the help of a motor and pestle using 20ml 80% acetone. The above preparation is subjected to centrifugation at 5000rpm for about 5 minutes and the supernatant is transferred to a volumetric flask of 100ml capacity. Grind the residue again using 20ml 80% acetone and collect the supernatant into the above volumetric flask. Repeat this procedure until the residue becomes colourelss. Wash the motor and pestle using 80% acetone and collect these washings into a flask. Make up the volume to 100ml using 80% acetone. Read the absorbance of this prepared solution at 645nm, 663nm against 80% acetone as blank.

$$\text{Chlorophyll a mg/g tissue} = 12.7\,(A663) - 2.69\,(A645) \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b mg/g tissue} = 22.9\,(A645) - 4.68\,(A663) \times \frac{V}{1000 \times W}$$

$$\text{Total chlorophyll mg/g tissue} = 20.2\,(A645) + 8.02\,(A663) \times \frac{V}{1000 \times W}$$

Photosynthetic Parameters (Abolghassem *et al*., 2018)

Photosynthesis related gas exchange parameters including net photosynthetic rate (P_N), stomatal conductance (g_S), intercellular CO_2 concentration (Ci), transpiration rate (E) and vapour pressure deficit (VPD) are analysed and measured using the LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA). The device is fitted with blue and red LEDs (LI-6400-02B) as a light source for the relevant photosynthetic measurements. All the leaf gas exchange properties are recorded under the sample chamber condition where photosynthetic photon flux density (PPFD) is at 1000 $\mu Mm^{-2}s^{-1}$, leaf temperature is at 25°C, and the CO_2 level is at 380μM CO_2 mol^{-1}. The leaf gas exchange measurements are measured on the second fully expanded leaf from the top of the plant during the period at 9:00 am –11:00 am. The data on each parameter presented an average of six readings taken from six plants from each treatment.

Analysis of Biochemical parameters

Plant materials for assessing the impact of Cr^{VI} stress at different concentration levels on various biochemical parameters are collected from young shoot parts of all treated plants and control of *Amaranthus dubius* on 25th day of heavy metal treatment.

Estimation of Total Carbohydrate by Phenol Sulphuric Acid Method (Hodge & Hofreite, 1962)

Reagents

Phenol 5%: Phenol 50g dissolved in water and diluted to 1 L

2.5 N HCl

Sulphuric acid 96%

Solid sodium carbonate

Standard Glucose: Stock- 100mg of glucose in 100ml water and working standards- 10ml of stock diluted to 100ml using distilled water.

Assay

100 mg of the sample is weighed in a boiling tube. Hydrolysed the sample in a boiling water bath for 3 hours with 5mL of 2.5 N HCl and then cooled to room temperature. Neutralized the sample with solid sodium carbonate until the effervescence ceases.

Made up the volume to 100ml and centrifuged. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes. Pipetted out 0.1 and 0.2mL of the sample solution in two separate test tubes and made up the volume in each tube to 1mL with water. Set a blank with 1mL of water. 1mL of phenol solution followed by 5mL of 96% sulphuric acid are added to each tube and shaken well. After 10 minutes, the tubes are again shaken and placed in a water bath at 25-30°C for 20 minutes. Read the color at 490nm. Calculate the amount of total carbohydrate present in the sample solution using the standard graph.

Estimation of Starch by Anthrone Method (Hodge & Hofreite, 1962)

Reagents

Ethanol 80%

Anthrone reagent: Dissolve 200mg Anthrone in 100ml ice cold 95% sulphuric acid

Perchloric acid 52%

Standard Glucose: Stock- 100mg of glucose in 100ml water and working standards- 10ml of stock diluted to 100ml using distilled water

Assay

Homogenize 0.1-0.5g of sample in hot 80% ethanol to remove sugars. Centrifuge and collect the residue. Wash the residue repeatedly in 80% ethanol till the washing does not give colour with anthrone reagent. Dry the residues well over a water bath. Add 5ml water and 6.5ml 52% perchloric acid into the residue and extract at 0^{o}C for 20 minutes. Centrifuge and collect the supernatant. Repeat this extraction procedure using fresh 52% perchloric acid and collect the supernatant to make up the volume to 100ml. Pipetted out 0.1 - 0.2mL of the supernatant sample solution and make up the volume to 1mL with water. Add 4ml of anthrone reagent and subject to heating for 8 minutes using a boiling water bath. Cool suddenly and read the absorbance at 630 nm.

Prepare the standards by taking 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes and make up the volume in each with water. Set a blank with 1mL of water. Calculate the glucose content in the sample using the standard graph and then multiply the value with a factor 0.9 to get starch content.

Estimation of Fat by Simplified Gravimetric Determination (Phillips *et al*., 1997**)**

Procedure:

- Samples of 1g are massed into a polypropylene centrifuge bottle. Sodium acetate is added so that the total volume of solution is 32 ml.
- Aliquots of methanol and chloroform are added, the bottles capped and shaken for 2 hours.
- Another aliquot of chloroform is added, and the bottles shaken again for 30 minutes.
- Finally, an aliquot of water is added, and the bottles are shaken a final time for 30 min.
- Centrifuge tubes which had been dried and massed are used to hold 20 ml aliquots of the chloroform layer. These are then centrifuged for 10 min, and allowed to set in 25°C water bath for 15 minutes.
- The samples are then evaporated to dryness under a nitrogen blanket, heated in a drying oven for 30 minutes, and cooled in a dessicooler for at least 30 minutes.
- Finally, they are massed and the total lipid content determined by:

Total Fat (g/100 g wet weight) = (W2-W1) ×Vc×100/ (VA×SW)

Where W2 is the weight of glass tube and dried extract (g)
W1 is the weight of empty dried glass tube (g)
Vc is the total volume of chloroform (ml)
VA is the volume of extract dried (ml)
SW is the weight of sample assayed (g).

Estimation of Total Protein Content (Lowry *et al*., 1951)

Extraction of Protein from Sample

Extraction is carried out with Phosphate buffer (pH 7.4). 1 g of the sample is weighed and crushed well with a mortar and pestle in 10 mL of the buffer. The sample is centrifuged and the supernatant is taken for protein estimation.

Estimation of Protein

Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and double distilled water in the test tube. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ ml. From these different dilutions, 0.2 ml protein solution is pipetted out to different test tubes and added 2 ml of alkaline copper sulphate reagent (analytical reagent). The solutions are mixed well and incubated at room temperature for 10 minutes. Then added 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubated for 30 min. The colorimeter is set at zero with blank and the optical density (measure the absorbance) is recorded at 660 nm. The absorbance is plotted against protein concentration to get a standard calibration curve. The absorbance of unknown sample is checked and the concentration of the unknown sample is determined using the standard curve.

Assessment of Antioxidant Enzyme Activity

a) Assay of Superoxide Dismutase (SOD) (Kakkar *et al.*, 1984)

The assay of Superoxide Dismutase is based on the inhibition of the formation of NADH-phenazine methosulphate - Nitroblue tetrazolium formazan. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (780µM)
5. Glacial acetic acid
6. n-butanol
7. Potassium phosphate buffer (50mM, pH 6.4)

Preparation of enzyme extract

The leaf samples (0.5g) are ground with 3ml of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants are used for the assay.

Assay:

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of Phenazine methosulphate, 0.3ml of Nitroblue tetrazolium, 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction is initiated by the addition of 0.2 ml of NADH. The mixture is incubated at 30°C for 90 seconds and arrested by the addition of 1ml of glacial acetic acid. The reaction mixture is then shaken with 4ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the Chromogen in the butanol layer is measured at 560nm in a spectrophotometer.

One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of Nitroblue tetrazolium (NBT) reduction in one minute.

b) Assay of Catalase (CAT) (Luck, 1974)

The UV absorption of hydrogen peroxide can be measured at 240nm and this absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

Reagents

1. Phosphate buffer: 0.067 M (pH 7.0)
2. Hydrogen peroxide (2mM) in phosphate buffer

Preparation of enzyme extract

20% homogenate is prepared in phosphate buffer. The homogenate is centrifuged and the supernatant is used for the enzyme assay.

Assay

H_2O_2-phosphate buffer (3ml) is taken in an experimental cuvette and this is followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units is recorded at 240nm in a spectrophotometer. The enzyme solution containing H_2O_2-free phosphate buffer served as control.

One enzyme unit is calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

c) Assay of Peroxidase (POD) (Reddy *et al*., 1995)

In the presence of the hydrogen donor pyrogallol or o-dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or o-dianisidine to a coloured product called purpurogalli can be followed spectrophotometrically at 430nm.

Reagents

1. Pyrogallol: 0.05 M in 0.1M phosphate buffer (pH 6.5)

2. H_2O_2: 1% in 0.1M phosphate buffer, pH 6.5

Preparation of enzyme extract

20% homogenate is prepared in 0.1M phosphate buffer (pH 6.5) from the young shoot portion of the plant, subjected to centrifugation and the supernatant is used for the assay.

Assay

To 3ml of pyrogallol solution, 0.1ml of the enzyme extract is added and the spectrophotometer is adjusted to read zero at 430 nm. To the test solution holding cuvette add 0.5ml of H_2O2 and mixed. The change in absorbance is recorded every 30 seconds up to 3 minutes in a spectrophotometer (Genesys 10-S, USA). One unit of peroxidase is defined as the change in absorbance/minute at 430nm.

Statistical Analysis

The mean value and standard deviation of various parameters in the study are determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSIONS

Chlorophyll a, Chlorophyll b and Total Chlorophyll content

The effects of Cr^{VI} stress on the content of chlorophyll in *Amaranthus dubius* are depicted in table 3.1. The data shows all the Cr^{VI} treatments have caused decrease in chlorophyll b and total chlorophyll content over the control in a progressive manner. The lowest reduction of 19.94% and 8.84% respectively for chlorophyll b and total chlorophyll content is obtained in the lowest Cr^{VI} stress of 1mg treatment and similarly, the highest reduction of 74.41% and 50.63% respectively is obtained in the highest Cr^{VI} stress of 70mg treatment. However, all Cr^{VI} treatments caused an increase in the chlorophyll a content over the control. The increases are progressive up to 10mg Cr^{VI} treatment and the highest content recorded is 0.433mg/g tissue which is about 26.79% increase over control while further increase in the concentration of Cr^{VI} treatments caused a progressive reduction and the lower most chlorophyll a content of 0.347mg/g tissue is obtained at 70mg Cr^{VI} treatment; but maintained a value still higher than control which is about 8.65% increase (figure 3.1).

Table 3.1: Effect of Cr^{VI} stress on the chlorophyll content in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Chlorophyll content (mg/g tissue)					
	Chlorophyll a	SD±	Chlorophyll b	SD±	Total Chlorophyll	SD±
0mg (C)	0.317	0.01	0.637	0.03	1.357	0.04
1mg	0.377	0.04	0.510	0.11	1.237	0.12
5mg	0.427	0.01	0.393	0.02	1.113	0.02
10mg	0.433	0.02	0.327	0.03	1.017	0.07
20mg	0.417	0.01	0.253	0.02	0.890	0.02
30mg	0.400	0.02	0.217	0.01	0.813	0.02
50mg	0.387	0.01	0.190	0.01	0.750	0.02
70mg	0.347	0.02	0.163	0.02	0.670	0.04

The reduction in the content of chlorophyll b and total chlorophyll in *A. dubius* plants induced by all the Cr^{VI} treatments are significant over control. The maximum reduction recorded due to 70mg Cr^{VI} treatment did not differ significantly over 50mg Cr^{VI} treatment with respect to total chlorophyll and 50mg & 30mg Cr^{VI} treatment with respect

to chlorophyll b, whereas the differences over rest of the treatments are significant (table 3.3 & 3.4). The highest increase in chlorophyll a content recorded by plants treated with 10mg Cr^{VI} are insignificant over 5mg & 20mg Cr^{VI} treated plants while the increase over control and other treated plants are significantly higher. The lowest increase in chlorophyll a content recorded with 70mg Cr^{VI} is insignificantly different over control and 1mg Cr^{VI} treatments while the differences with others are significantly higher (table 3.2). The reduction in the content of chlorophyll pigments inferred in *A. dubius* plants in the study can be attributed to the inhibitory action of hexavalent Cr stress on the specific enzyme protochlorophyllide reductase, which is essential for the reduction of protochlorophyll to chlorophyll during the biosynthetic pathways of photosynthetic pigments (De Filippis & Pallaghy, 1994). Further, the depletion of chlorophyll content can also be due to toxicity of Cr^{VI} on the development of chloroplast and the replacement of central Mg^{2+} ion of chlorophyll molecule by the Cr^{VI} (Vajpayee *et al*., 2000; Küpper *et al*., 2002)

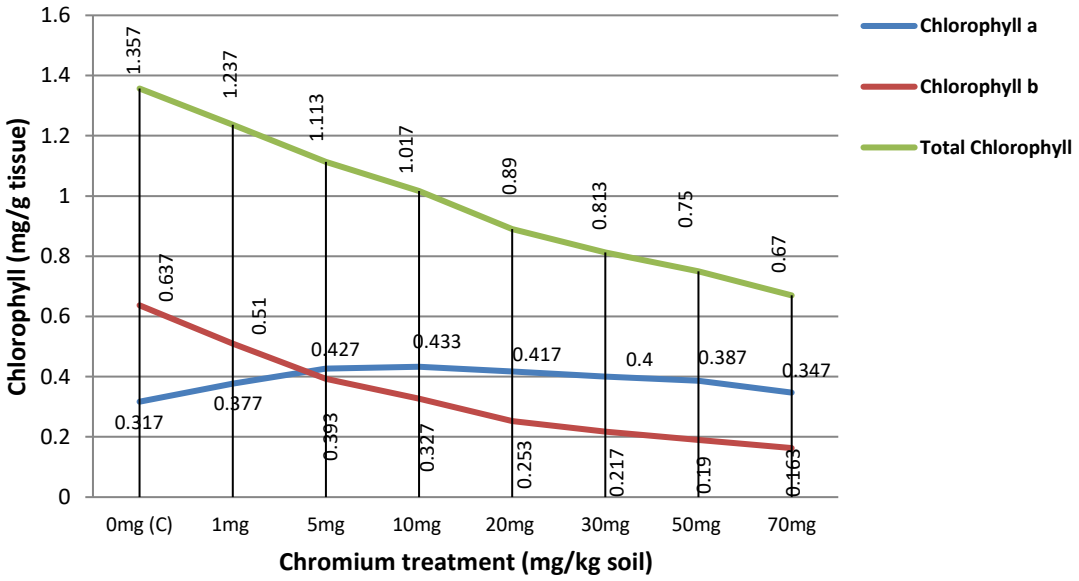

Figure 3.1: The impact of Cr^{VI} stress on the chlorophyll content in *A. dubius* plant

Table 3.2: Duncan analysis of chlorophyll a content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
1 (Control)	.317					
8 (70mg)	.347	.347				
2 (1mg)		.377	.377			
7 (50mg)			.387	.387		
6 (30mg)			.400	.400	.400	
5 (20mg)				.417	.417	.417
3 (5mg)					.427	.427
4 (10mg)						.433
Sig.	.056	.056	.147	.067	.100	.293

Means for groups in homogeneous subsets are displayed.

Table 3.3: Duncan analysis of chlorophyll b content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8	.163				
7	.190	.190			
6	.217	.217			
5		.253			
4			.327		
3			.393		
2				.510	
1					.637
Sig.	.150	.091	.064	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 3.4: Duncan analysis of total chlorophyll content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
8	.670						
7	.750	.750					
6		.813	.813				
5			.890				
4				1.017			
3					1.113		
2						1.237	
1							1.357
Sig.	.088	.170	.101	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Photosynthetic rate (P_N)

With an exception of lowest concentration of 1mg Cr^{VI}/kg soil treatment, all the Cr^{VI} concentration treatments have caused a reduction in net photosynthetic rate. The impact of different concentrations of Cr^{VI} stress on net photosynthetic rate in *Amaranthus dubius* is depicted in the table 3.5. The highest average photosynthetic rate of 10.08 µmol CO_2m^2/sec is obtained in *A. dubius* plants treated with 1mg Cr^{VI}/kg soil and recorded an average increase of 8.83% over control. On further increase in Cr^{VI} stress by increasing the concentration of treatment, the result revealed a progressive reduction in net photosynthetic rate (figure 3.2). The lowest photosynthetic rate of 2.66 µmol CO_2m^2/sec is obtained in the highest concentration of 70mg Cr^{VI}/kg soil which recorded an average decrease of 71.06% over the control.

Table 3.5: Photosynthetic rate of *A. dubius* against different concentrations of Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Photosynthetic rate (PR) µmol CO_2m^2/sec	SD±
0mg (Control)	9.19	2.12
1mg	10.08	1.78
5mg	6.31	0.43
10mg	5.71	0.48
20mg	4.70	0.75
30mg	3.98	0.73
50mg	3.44	0.72
70mg	2.66	0.83

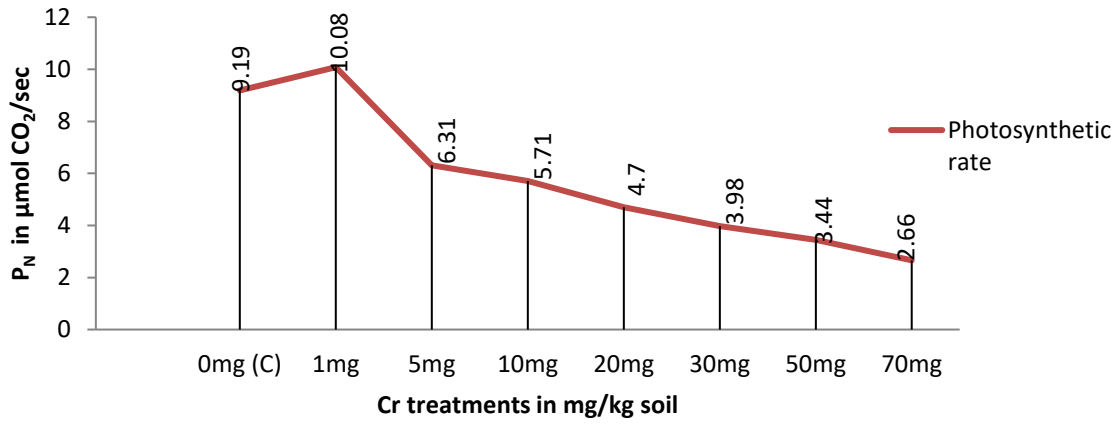

Figure 3.2: Rate of photosynthesis in *A. dubius* against different concentrations of Cr^{VI} stress

The lowest photosynthetic rate obtained at 70mg Cr^{VI} stress is found not to have significant differences over the rate obtained at 50mg and 30mg Cr^{VI} stress while the differences over the control and rest of the Cr^{VI} stress treatments have revealed significant reduction. The highest photosynthetic rate obtained at1mg Cr^{VI} stress differed only insignificantly higher over control whereas the differences over all other treated plants are significantly higher (table 3.6).

Table 3.6: Duncan analysis of net photosynthetic rate in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	2.6583				
7 (50mg)	3.4433	3.4433			
6 (30mg)	3.9800	3.9800			
5 (20mg)		4.7033	4.7033		
4 (10mg)			5.7100	5.7100	
3 (5mg)				6.3133	
1 (Control)					9.1917
2 (1mg)					10.0767
Sig.	.064	.077	.134	.365	.186

Means for groups in homogeneous subsets are displayed.

There are reports on inhibition of growth and productivity in plants which are growing under heavy metal stress due to disturbances in electron transport system and reduction in net photosynthetic rate (Desmet *et al*., 1975; Foy *et al*., 1978; Baszyński *et al*., 1981). The present study observations are in agreement with these reports. The reduction in net photosynthetic rate and growth recorded in *A. dubius* plants subjected to hexavalent chromium stress and toxicity can be attributed to their inhibitory action on ATP production and energy level of cells by affecting the primary photosynthetic reactions. This in turn may be the result of disturbances by hexavalent chromium toxicity on electron carriers and the flow rate of electron from water to PS-II reaction centre (Prasad *et al*., 1991). The availability of heavy metals in toxic level in the growing medium can cause disruption in many vital activities in plants such as biosynthesis of chlorophyll molecules and activities of many relevant enzymes by interfering with the functional role of certain essential metals which are acting as cofactors or stimulating agent in many physiological and metabolic processes. This in turn may adversely affect the growth and

productivity by retarding the rate of photosynthesis and other physiological processes in plants (Hossain, 2012; Ali, 2013).

Stomatal conductance (gs)

Similar trend as in the case of photosynthetic rate is recorded with respect to stomatal conductance. The details of stomatal conductance in *Amaranthus dubius* plants treated with different concentrations of Cr^{VI} stress are depicted in the table 3.7. The highest stomatal conductance in the study is recorded in *A. dubius* plants treated with lowest Cr^{VI} stress of 1mg Cr^{VI}/kg soil treatment while the maximum reduction is recorded with highest Cr^{VI} stress of 70mg Cr^{VI}/kg soil treatment. Similar to the photosynthetic rate, the stomatal conductance also recorded a progressive reduction from the highest stomatal conductance of 0.077 m mol $m^{-2}s^{-1}$ to the lowest value of 0.023 m mol $m^{-2}s^{-1}$ obtained along with progressive increase in Cr^{VI} stress from 1mg Cr^{VI}/kg soil to 70mg Cr^{VI}/kg soil treatment (figure 3.3).

Table 3.7: Stomatal conductance of *A. dubius* against different concentrations of Cr^{VI}

Treatment Cr^{VI} / kg soil	Stomatal conductance m mol $m^{-2}s^{-1}$	SD±
0mg (Control)	0.075	0.019
1mg	0.077	0.007
5mg	0.056	0.003
10mg	0.052	0.009
20mg	0.042	0.008
30mg	0.034	0.004
50mg	0.031	0.006
70mg	0.023	0.005

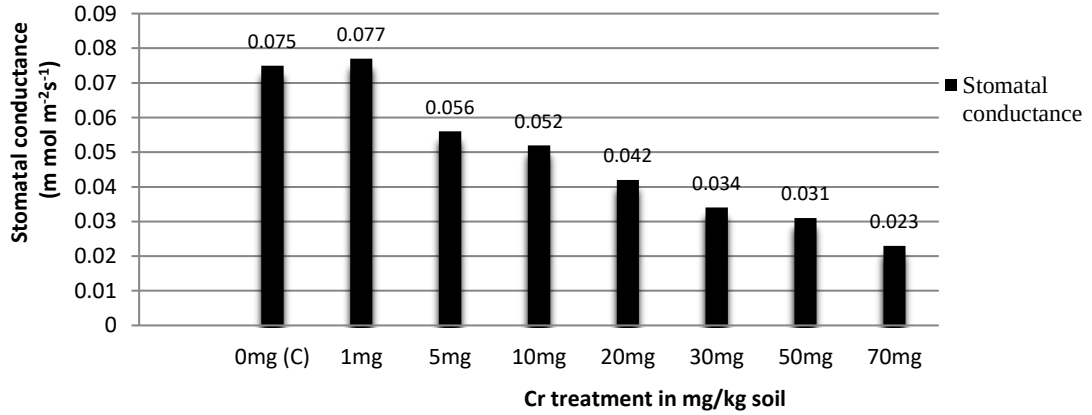

Figure 3.3: Stomatal conductance in *A. dubius* plants in response to Cr^{VI} stress

The increase in stomatal conductance of 2.6% over control recorded in 1mg Cr^{VI} treated plants is insignificant. The differences due to reduction in stomatal conductance recorded by plants treated with all the Cr^{VI} stress treatments are significantly lower with the highest difference of 69.33% decrease over control being recorded in70mg Cr^{VI} treated plants (table 3.8).

Table 3.8: Duncan analysis of stomatal conductance in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	.023				
7 (50mg)	.031	.031			
6 (30mg)		.034			
5 (20mg)			.042		
4 (10mg)				.052	
3 (5mg)				.056	
1 (Control)					.075
2 (1mg)					.077
Sig.	.056	.536	1.000	.251	.566

Means for groups in homogeneous subsets are displayed.

Liu *et al*. in 2008 observed a decrease in stomatal conductance followed by fall in rate of CO_2 assimilation in plants while evaluating the impact of Cr^{VI} stress on fluorescence parameters and these observations are in similar lines with present study inferences. The reduction in stomatal conductance under Cr^{VI} stress inferred in the study might be the result of toxicity induced water imbalance and poor stomatal opening caused by loss in turgor pressure inside the guard cells of stomata (Barbosa *et al*., 2007).

Intercellular CO2 concentration (C_i)

The intercellular CO_2 concentrations in *Amaranthus dubius* plants treated with different concentrations of Cr^{VI} stress is found to respond in similar lines with stomatal conductance and net photosynthetic rate and the details are depicted table 3.9. The highest CO_2 concentration of 156.27 µmol CO_2 $mole^{-1}$ recorded in 1mg Cr^{VI} treated plants is about 0.88% increase over control. Further enhancement in Cr^{VI} stress caused a progressive reduction in CO_2 concentration and the lowest CO_2 concentration of

58.7µmol CO_2 $mole^{-1}$ is recorded in 70mg Cr^{VI} treated plants, which is about 62.10% reduction over control (figure 3.4).

Table 3.9: Intercellular CO_2 concentration of *A. dubius* plants treated with Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Intercellular CO_2 concentration µmol CO_2 $mole^{-1}$	SD±
0mg (Control)	154.9	24.83
1mg	156.27	24.06
5mg	140.38	12.93
10mg	139.67	12.98
20mg	115.1	28.24
30mg	99.3	18.23
50mg	76.13	19.64
70mg	58.7	16.12

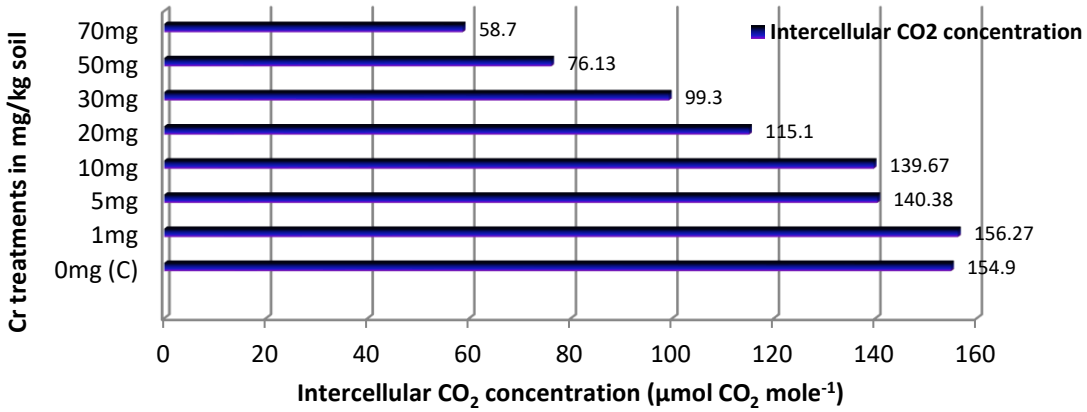

Figure 3.4: Intercellular CO_2 concentration in *A. dubius* in response to Cr^{VI} stress

The increased intercellular CO_2 concentration recorded by1mg Cr^{VI} treated plants is insignificant over the control and 5mg & 10mg Cr^{VI} treated plants whereas the reductions in CO_2 concentration recorded by 20mg, 30mg, 50mg and 70mg Cr^{VI} treated plants are significantly lower when compared to control and 1mg, 5mg & 10mg Cr^{VI} treated plants. The difference in CO_2 concentration recorded by 70mg Cr^{VI} treated plants insignificantly differed over 50mg Cr^{VI} treated plants while the differences over control and other treated plants are significant (table 3.10).

Table 3.10: Duncan analysis of Intercellular CO_2 concentration in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	58.70			
7 (50mg)	76.13	76.13		
6 (30mg)		99.30	99.30	
5 (20mg)			115.10	
4 (10mg)				139.67
3 (5mg)				140.38
1 (Control)				154.90
2 (1mg)				156.27
Sig.	.145	.055	.186	.205

Means for groups in homogeneous subsets are displayed.

The decrease in CO_2 assimilation due to hexavalent chromium (Cr^{VI}) stress clearly revealed the toxic effect of Cr^{VI} in *A. dubius*. The study indicates the existence of a negative correlation between the degree of Cr^{VI} stress and intercellular CO_2 concentrations, which is evident from the progressive reduction in CO_2 concentrations along with increasing Cr^{VI} stress. The reduction in intercellular CO_2 concentrations clearly revealed the toxic and inhibitory effect of Cr^{VI} stress on stomatal conductance which affects the gas exchange activities (Henriques, 2010; Ahmad *et al*., 2011). Further it can also be attributed to negative interference of Cr^{VI} with biosynthesis of photosynthetic pigments and disruption in the activities of enzymes involved in carbon fixation (Larcher, 1995; Vajpayee *et al*., 2000).

Transpiration rate (E)

The rate of transpiration in *Amaranthus dubius* plants obtained against different concentrations of Cr^{VI} stress revealed variations and the details are shown in table 3.11. A progressive reduction in rate of transpiration is observed in the study with the highest E value of 3.92 m mol H_2O m^2/sec being recorded in lowest stress of 1mg Cr^{VI} treatment, which is about 2.04% increase over control and the lowest E value of 1.72 m mol H_2O m^2/sec recorded in highest stress of 70mg Cr^{VI} treatment, which is about 55.21% decrease over control (figure 3.5).

Table 3.11: Transpiration rate in *A. dubius* treated with different concentrations of Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Transpiration rate m mol H_2O m^2/sec	SD±
0mg (Control)	3.84	0.27
1mg	3.92	0.14
5mg	3.07	0.11
10mg	2.88	0.32
20mg	2.20	0.77
30mg	2.03	0.47
50mg	1.97	0.21
70mg	1.72	0.34

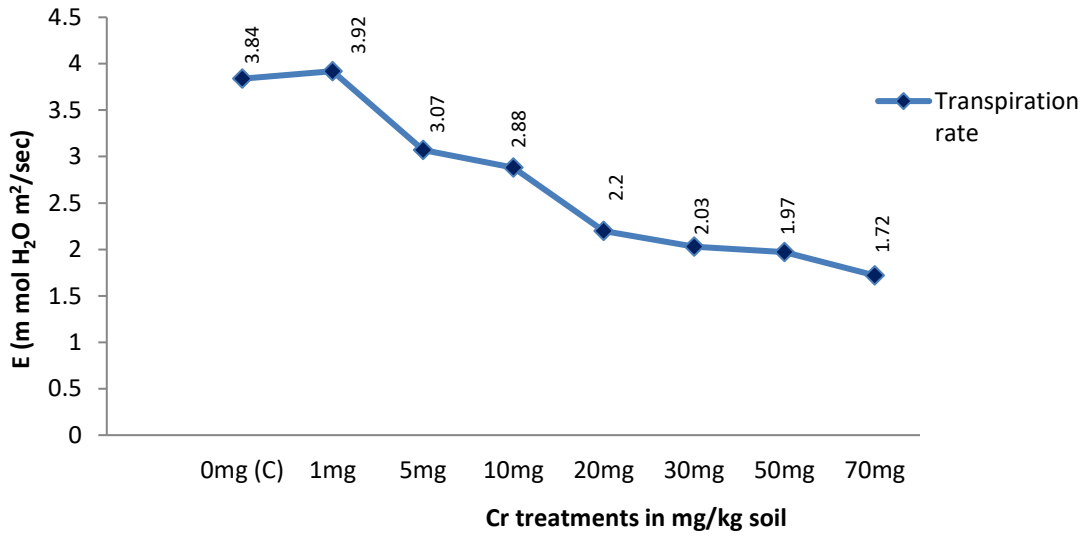

Figure 3.5: Transpiration rate in *A. dubius* against different concentrations of Cr^{VI} stress

The higher rate of transpiration obtained in 1mg Cr^{VI} treated plants over control is insignificant whereas this rate differed significantly higher over rest of the treated plants. The lowest E value induced due to 70mg Cr^{VI} treatment did not differ significantly over the E values obtained due to 20mg, 30mg and 50mg Cr^{VI} treatment while differences over control and 1mg, 5mg & 10mg Cr^{VI} treated plants are significantly lower (table 3.12).

Table 3.12: Duncan analysis of Transpiration rate in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	1.7150		
7 (50mg)	1.9683		
6 (30mg)	2.0333		
5 (20mg)	2.1983		
4 (10mg)		2.8750	
3 (5mg)		3.0683	
1 (Control)			3.8383
2 (1mg)			3.9183
Sig.	.052	.391	.721

Means for groups in homogeneous subsets are displayed.

The accumulation of chromium in toxic level and related water stress could disturb the functioning and efficacy of the stomata and this can affect the transpiration rate as well as other gas exchange activities. The water stress can be attributed to the toxicity induced disturbances in the passive movement of water flow through roots which is established based on the water potential gradient by transpiration processes (Barceló & Poschenrieder, 1990). Davies in 2002 reported that, 71% reduction in transpiration rate is induced by chromium toxicity in sunflower plants. The present investigation also obtains similar observation and recorded maximum of 55.21% decrease over control at 70mg Cr^{VI} stress. The Cr^{VI} toxicity induced water stress followed by the production and accumulation of abscisic acid (ABA) molecules and their involvement in signal pathways to control water loss by causing stomatal closure might be the key factor for reduction of transpiration rate (Rucińska, 2016). Further, the toxicity induced reduction in leaf parameters like decrease in leaf size and thickness of lamina, reduction in stomatal density and stomatal aperture size, intercellular spaces etc. may also have contributed towards the fall of transpiration rate in the study.

Vapour pressure deficit (VPD)

Different concentrations of Cr^{VI} stress in *Amaranthus dubius* caused variations in vapour pressure deficit (table 3.13). With the exception of 1mg Cr^{VI} treatment, all the Cr^{VI} concentration treatments caused an increase in vapour pressure deficit (VPD) over control progressively which ranges from VPD of 4.89 in the lower concentration of 5mg Cr^{VI} to VPD of 6.02 in the highest concentration of 70mg Cr^{VI} (figure 3.6). With respect to 1mg Cr^{VI} treatment, a decrease of 4.03% is recorded over control.

Table 3.13: Vapour pressure deficit (VPD) in *A. dubius* treated with different concentrations of Cr^{VI} stress

Treatment Cr^{VI} / kg soil	Vapour pressure deficit (VPD)	SD±
0mg (Control)	4.47	0.22
1mg	4.29	0.29
5mg	4.89	0.11
10mg	4.89	0.46
20mg	5.38	0.46
30mg	5.65	0.41
50mg	5.93	0.29
70mg	6.02	0.28

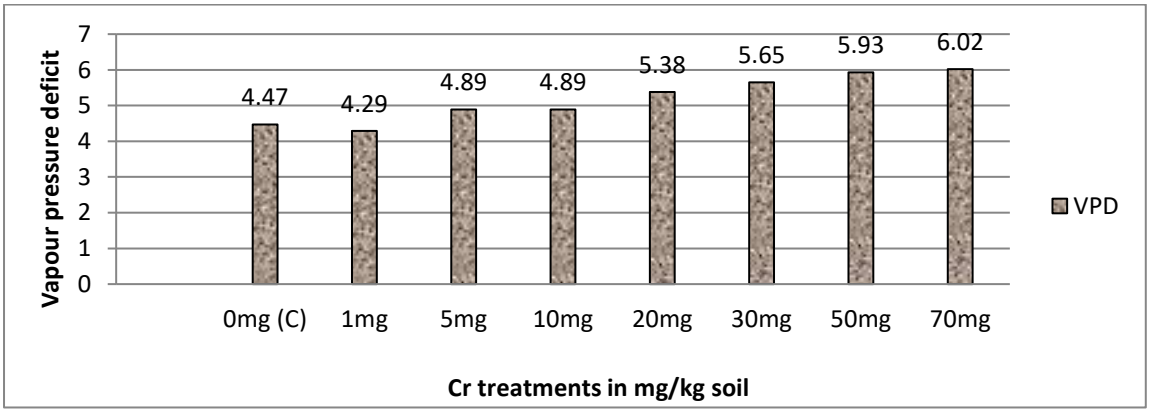

Figure 3.6: Vapour pressure deficit in *A. dubius* against different concentrations of Cr^{VI} stress

The decrease in vapour pressure deficit obtained in 1mg Cr^{VI} treated plants over control plants is only insignificantly lower. However, the increase in vapour pressure deficit recorded by all other Cr^{VI} concentration treated plants differed significantly higher over control and1mg Cr^{VI} treated plants. The highest VPD value obtained by 70mg Cr^{VI}

treated plants did not differ significantly over 30mg and 50mg Cr^{VI} treated plants while the increase over control and other treated plants are significant (table 3.14).

Table 3.14: Duncan analysis of Vapour pressure deficit (VPD) in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
2 (1mg)	4.2933			
1 (Control)	4.4700			
4 (10mg)		4.8867		
3 (5mg)		4.8900		
5 (20mg)			5.3783	
6 (30mg)			5.6533	5.6533
7 (50mg)				5.9317
8 (70mg)				6.0217
Sig.	.368	.986	.164	.079

Means for groups in homogeneous subsets are displayed.

Under water stress condition, the unregulated transpiration gradually lowers leaf water potential and automatically increases the vapour pressur deficit (VPD) causing negative effect on stomatal conductance which is probably triggered by the generation of ABA in the leaves (Bunce, 1997). This in turn is capable of causing reduction in transpiration rate. The present study clearly revealed the existence of a positive correlation between the Cr^{VI} stress and VPD and a negative correlation of VPD with stomatal conductance and transpiration rate. The increase in VPD recorded in *A. dubius* plants along with increase in Cr^{VI} stress which is in parallel with the inhibition of growth inferred in the study might be the results of above-mentioned correlations.

Total fat, Starch and Total Carbohydrates

The effects of different levels of Cr^{VI} stress on the content of total fat, starch and carbohydrates are depicted in the table 3.15. The results revealed all the Cr^{VI} stress levels, with certain exceptions with respect to the lowest stress of 1mg Cr^{VI}, caused decrement in the content of fat, starch and carbohydrates progressively along with the increase of Cr^{VI} stress (figure 3.7, 3.8 & 3.9). The lowest content of fat, starch and carbohydrates are

recorded at highest Cr^{VI} stress of 70mg treatment and are 14.13g, 0.05mg and 90mg respectively, which are 46.68%, 61.54% and 35.58% reduction respectively over the control. The lowest fat content obtained is significantly lower over the control and other stress treatments whereas, the lowest content obtained for starch and carbohydrates did not differ significantly over the content obtained with 50mg Cr^{VI} stress, while the differences over rest of the treatments are significant. The differences in the content of fat, starch and carbohydrates induced by the initial level stresses like 1mg, 5mg and 10mg Cr^{VI} are insignificant over control and amongst them (table 3.16, 3.17 & 3.18).

Table 3.15: Effects of Cr^{VI} stress on total fat, starch and carbohydrate content in *A. dubius*

Treatment Cr (mg/kg soil)	**Total fat content** (g/100g)	**SD±**	**Starch** (mg/g)	**SD±**	**Total Carbohydrate** (mg/g)	**SD±**
0mg ©	26.50	0.36	0.13	0.01	139.7	1.5
1mg	26.47	0.45	0.13	0.01	139.7	2.5
5mg	26.20	0.60	0.12	0.01	138.3	2.5
10mg	25.73	0.65	0.11	0.02	135.7	2.3
20mg	20.93	0.58	0.09	0.01	125.0	3.6
30mg	19.07	1.40	0.08	0.01	114.0	6.0
50gm	15.67	1.33	0.06	0.01	91.3	6.5
70mg	14.13	0.59	0.05	0.02	90.0	4.0

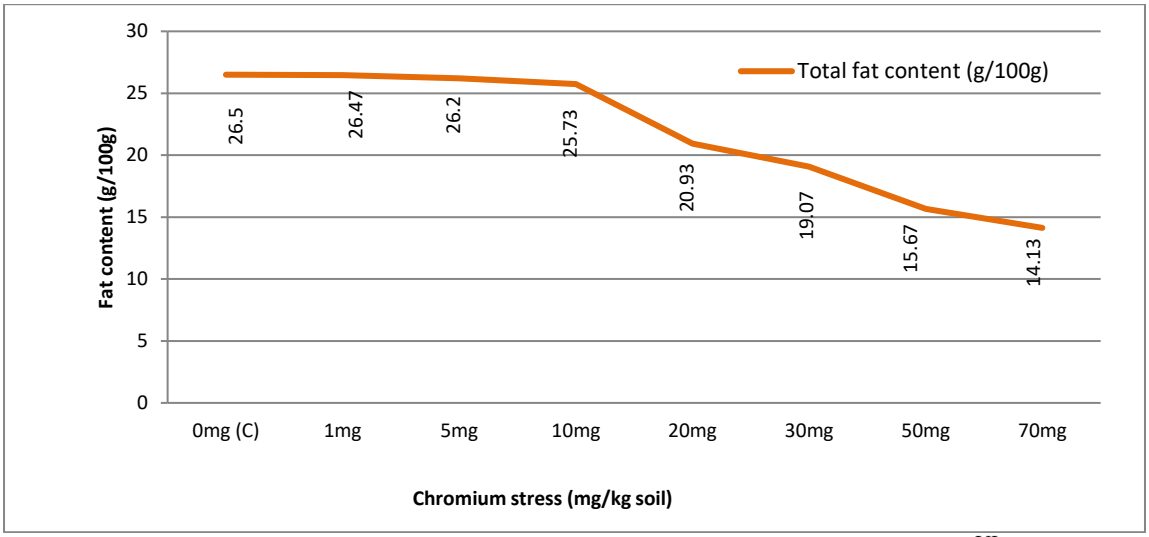

Figure 3.7: Total fat content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 3.16: Duncan analysis of total fat content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	14.13				
7 (50mg)		15.67			
6 (30mg)			19.07		
5 (20mg)				20.93	
4 (10mg)					25.73
3 (5mg)					26.20
2 (1mg)					26.57
1 (Control)					26.5
Sig.	1.000	1.000	1.000	1.000	.315

Means for groups in homogeneous subsets are displayed

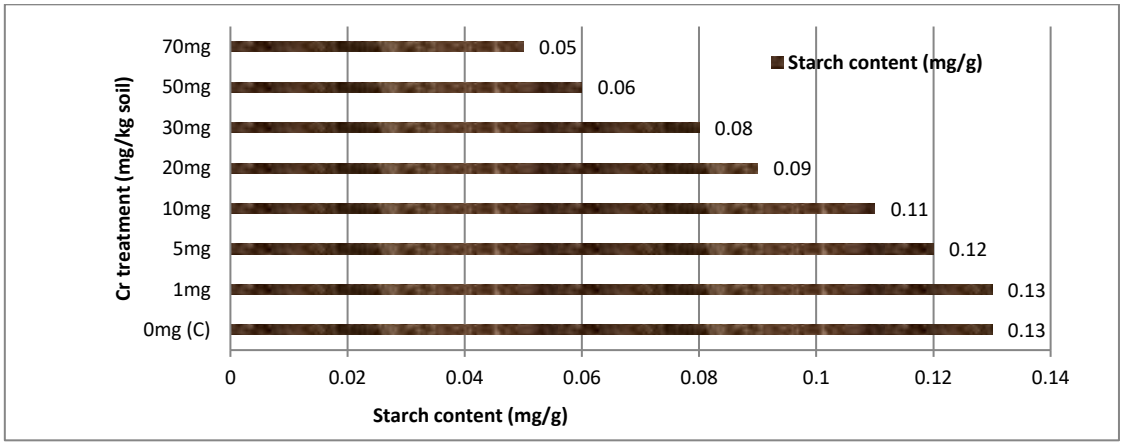

Figure 3.8: Starch content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 3.17: Duncan analysis of starch content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	.0500		
7 (50mg)	.0567		
6 (30mg)		.0800	
5 (20mg)		.0867	
4 (10mg)			.1100
3 (5mg)			.1233
2 (1mg)			.1267
1 (Control)			.1300
Sig.	.503	.503	.075

Means for groups in homogeneous subsets are displayed

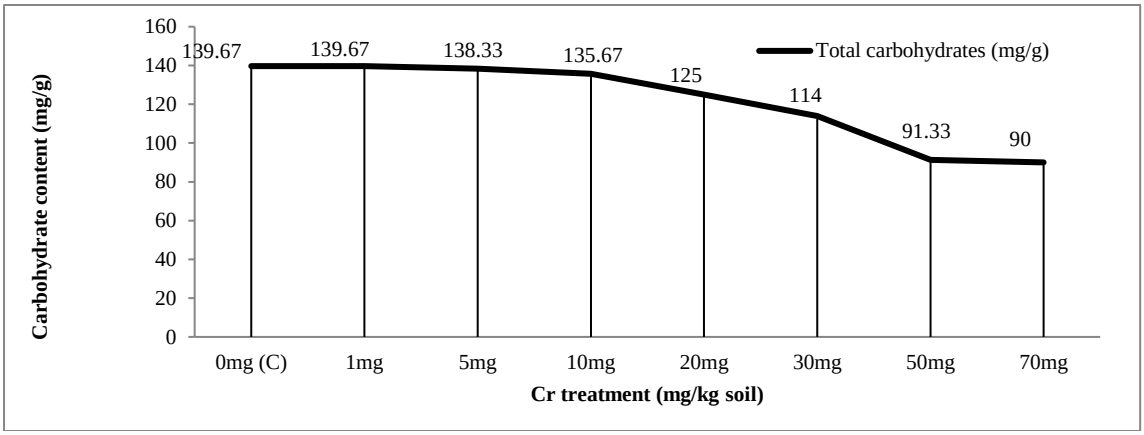

Figure 3.9: Total carbohydrate content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 3.18: Duncan analysis of total carbohydrate content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	90.0			
7 (50mg)	91.3			
6 (30mg)		114.0		
5 (20mg)			125.0	
4 (10mg)				135.7
3 (5mg)				138.3
1 (Control)				139.7
2 (1mg)				139.7
Sig.	.688	1.000	1.000	.276

Means for groups in homogeneous subsets are displayed.

The heavy metals have strong impact on the growth promoting factors of leaves such as starch and total carbohydrate content in vegetable crops due to oxidative stress. The damage that occurred to chloroplast and the disturbances in the photosynthetic electron transport chain caused by Cr^{VI} toxicity induced production of free radicals like superoxide ($O2^{\cdot-}$), hydroxyl (OH^{-}), or non-free radical species like singlet oxygen (O^2) and hydrogen peroxide (H_2O_2) might have contributed towards the reduction in starch and total carbohydrate content in *Amaranthus dubius* plants (Sandalio *et al.*, 2001;

Khanna, 2012). Further, it can also attribute to the drop in the content of photosynthetic pigments like carotenoids and chlorophylls and the interference of Cr^{VI} with the role of certain enzymes involved in the metabolism of fat, starch and other carbohydrates (Gawęda, 2007; Nagor & Vyas, 1997).

Total protein content

The impact of different levels of Cr^{VI} stress on the total protein content in *Amaranthus dubius* is depicted in the table 3.19. An increase in the content of total protein is recorded in the initial level concentrations of 1mg and 5mg Cr^{VI} which are 0.22% and 0.94% increase respectively over control. Further enhancement in the Cr^{VI} stress caused a progressive decrease over control and the maximum reduction that is obtained in the highest stress of 70mg Cr^{VI} is about 67.28% decrease over control (figure 3.10). The increases in the total protein content due to initial level concentrations of 1mg and 5mg and the decreases due to 10mg and 20mg Cr^{VI} concentrations are insignificant over the protein content of control while the reductions due to higher level concentrations of 30mg, 50mg and 70mg are significant. The highest reduction that is recorded by 70mg Cr^{VI} treated plants did not differ significantly over 50mg Cr^{VI} treated plants whereas the differences over rest of the treatments are significant (table 3.20).

Table 3.19: The effect of Cr^{VI} stress on the total protein content in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Total Protein (mg/g)	SD±
0mg (Control)	18.03	0.15
1mg	18.07	0.15
5mg	18.20	0.17
10mg	17.80	0.20
20mg	17.13	0.59
30mg	11.40	1.70
50mg	6.57	1.15
70mg	5.90	0.60

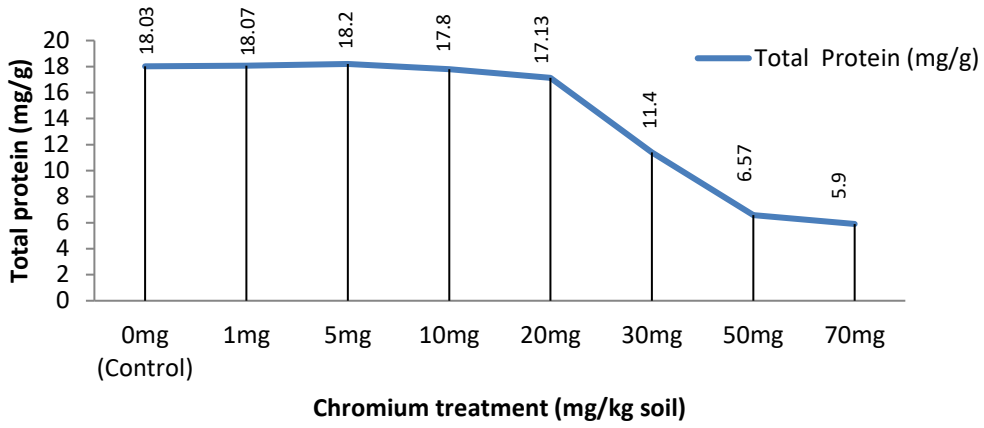

Figure 3.10: Total protein content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 3.20: Duncan analysis of total protein content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	5.90		
7 (50mg)	6.57		
6 (30mg)		11.40	
5 (20mg)			17.13
4 (10mg)			17.80
1 (Control)			18.03
2 (1mg)			18.07
3 (5mg)			18.20
Sig.	.319	1.000	.155

Means for groups in homogeneous subsets are displayed.

The lower-level concentrations of heavy metal stress have been reported to induce a variety of stress tolerating proteins in plants (Shah & Dubey, 1997; Lee *et al*., 2010). The production of such proteins including proline as a mechanism to tolerate stress under mild Cr^{VI} toxicity might be the reason leading to an overall increase in protein content of *A. dubius* plants when treated with 1mg and 5mg Cr^{VI}/kg soil. Whereas, the significant reduction in total protein content observed at higher level concentrations of Cr^{VI} can be attributed to the inhibition of protein synthesis due to disturbance in nitrogen metabolism under severe Cr toxicity (Auda & Ali, 2010; Solomonson & Barber, 1990; Panda & Choudhury, 2005a). Further, it can also be attributed to Cr toxicity induced lipid

peroxidation and fragmentation of proteins along with protein degradation process under higher protease and other catabolic enzyme activities.

Antioxidant enzymes activity

All the Cr^{VI} stress levels have caused differences in the activities of antioxidant enzymes Superoxide Dismutase (SOD), Catalase (CAT) and Peroxidase (POD) over the control. The details of antioxidant enzyme activities in *Amaranthus dubius* plants in response to different levels of Cr^{VI} stress is depicted in the table 3.21. The activity increased progressively up to 20mg Cr^{VI} stress with respect to SOD and CAT and then started to decrease (figure 3.11 & 3.12) while with respect to POD activity, the increase is progressive up to 50mg Cr^{VI} stress and then recorded a decrease at 70mg Cr^{VI} (figure 3.13).

Table 3.21: Effects of different levels of Cr^{VI} stress on antioxidant enzymes activity in *A. dubius*

Treatment Cr^{VI} / kg soil	Antioxidant enzyme activity					
	SOD activity (U/mg)	SD±	CAT activity (U/mg)	SD±	POD activity (U/mg)	SD±
0mg (Control)	0.98	0.02	0.023	0.01	88.27	5.95
1mg	1.00	0.01	0.027	0.01	92.19	4.71
5mg	1.01	0.01	0.090	0.03	113.43	2.77
10mg	1.02	0.01	0.130	0.02	119.55	2.41
20mg	1.03	0.01	0.150	0.02	121.81	1.19
30mg	0.89	0.02	0.147	0.02	124.29	0.49
50mg	0.49	0.02	0.090	0.02	124.44	0.30
70mg	0.46	0.02	0.067	0.02	120.16	1.56

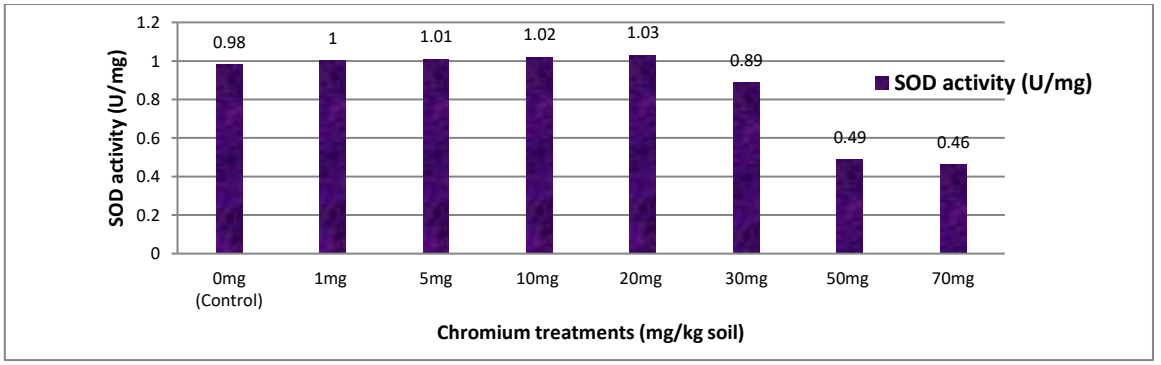

Figure 3.11: Impact of Cr^{VI} stress on Superoxide Dismutase (SOD) activity in *A. dubius*

Table 3.22: Duncan analysis of SOD activity in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
8	.46					
7		.49				
6			.89			
1				.98		
2				1.00	1.00	
3					1.01	1.01
4					1.02	1.02
5						1.03
Sig.	1.000	1.000	1.000	.348	.073	.111

Means for groups in homogeneous subsets are displayed

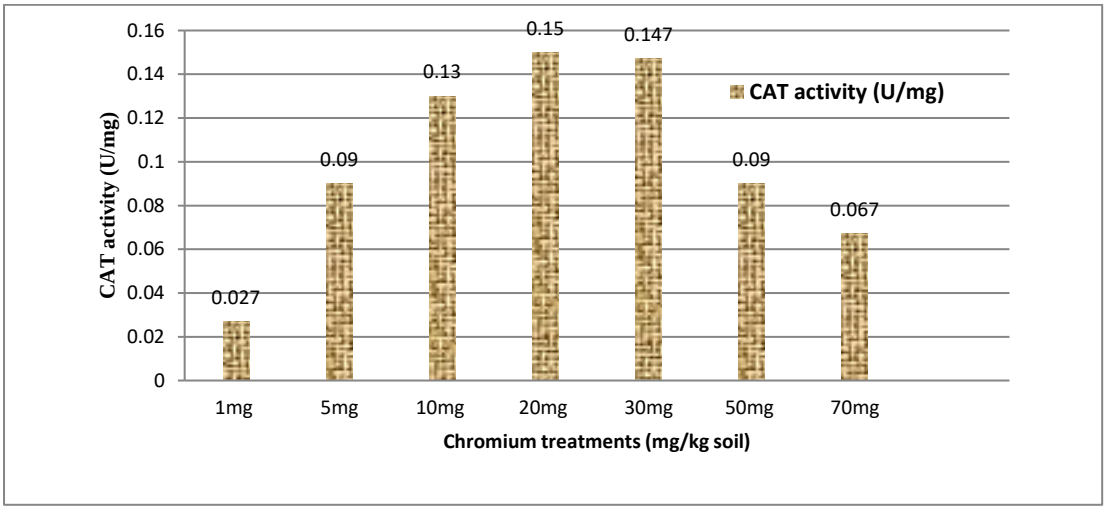

Figure 3.12: Impact of Cr^{VI} stress on Catalase (CAT) activity in *A. dubius*

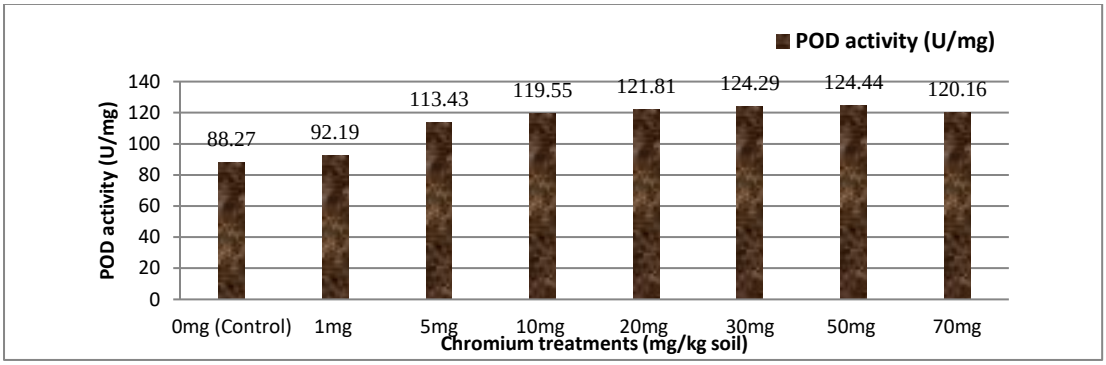

Figure 3.13: Impact of Cr^{VI} stress on Peroxidase (POD) activity in *A. dubius*

The highest SOD activity of 1.03U/mg and CAT activity of 0.150U/mg recorded at 20mg Cr^{VI} stress did not differ significantly over the activities at 10mg & 5mg and 10mg

&30mg Cr^{VI} stress respectively for SOD and CAT, whereas the highest POD activity of 124.44U/mg recorded at 50mg Cr^{VI} stress did not differ significantly over 10mg, 20mg, 30mg & 70mg Cr^{VI} stress. Similarly, the lowest SOD activity recorded at 70mg Cr^{VI} stress differed significantly lower over the activities of all the Cr^{VI} stresses and control whereas the lowest CAT and POD activity recorded in control plants did not differ significantly over the activity of 1mg Cr^{VI} stress while the differences over the rest are significant (Figure 3.22, 3.23 & 3.24).

Table 3.23: Duncan analysis of CAT activity in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
1	.023		
2	.027		
8		.067	
3		.090	
7		.090	
4			.130
6			.147
5			.150
Sig.	.825	.154	.219

Means for groups in homogeneous subsets are displayed.

Table 3.24: Duncan analysis of POD activity in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
1	88.270		
2	92.19		
3		113.43	
4			119.55
8			120.16
5			121.81
6			124.29
7			124.44
Sig.	.137	1.000	.096

Means for groups in homogeneous subsets are displayed.

The increase in the activity of antioxidant enzymes in *A. dubius* under comparatively low or medium concentrations of Cr^{VI} stress can be attributed to the increased transcript levels of the genes encoding antioxidant enzymes to mitigate the harmful effects of free radicals. The inference in the present study suggest, reactive oxygen species (ROS) could be induced by phytotoxic concentrations of Cr^{VI} leading to increased CAT activities

which play a crucial role in detoxification of elevated concentrations of Cr^{VI} possibly via lignifications and physical barrier formation (Sai *et al*., 2009). The decline in antioxidant enzyme activity at higher Cr^{VI} stresses may result from the inactivation of the enzyme by ROS like H_2O_2, which is produced in different cellular compartments or from a number of non-enzymatic and enzymatic processes in cells (Saffar *et al*., 2009). The results indicate that after the progressive increase in the activity of various antioxidant enzymes in response to Cr^{VI} stress, the lowest activity of SOD is recorded at the highest Cr^{VI} stress of 70mg Cr^{VI}/kg soil treatment which falls below the activity level of control plants and it is about 53.06% decrease over control. However, with respect to the CAT and POD activities, although their activities have also started to decline at higher levels, the lower activity recorded at highest Cr^{VI} stress of 70mg Cr^{VI} are maintained higher over the control plants and are 65.67% and 26.54% respectively higher than control. The lipid peroxidation and cell damage induced by severe Cr^{VI} toxicity may inhibit the transcript levels of genes encoding antioxidant enzymes and this in turn may reduce the antioxidant defense mechanism against the oxidative damage caused by ROS, as reflected by the drastic reduction of vigor in higher Cr^{VI} stressed plants (Omosun *et al*., 2008).

CONCLUSION

The responses of various physiological and biochemical parameters obtained against different levels of hexavalent chromium (Cr^{VI}) stress indicates Cr^{VI} toxicity induced disruption in the physiological and biochemical processes of *Amaranthus dubius* plant. The parameters such as chlorophyll b, total chlorophyll, net photosynthetic rate (P_N), stomatal conductance (g_S), intercellular CO_2 concentration (Ci), transpiration rate (E), content of starch, total carbohydrate, fat and protein generally recorded a progressive decrease in their values in parallel with progressive increase in the Cr^{VI} stress, with certain exceptions in the initial low stress level. However, antioxidant enzyme activities of POD and CAT and photosynthetic parameters such as chlorophyll a content, vapour pressure deficit etc. recorded a progressive increase in their values up to certain level of Cr^{VI} stress and then tended to decline at higher stress level but maintained the values higher over the control. These changes induced under Cr^{VI} stress on various physiological and biochemical parameters of *A. dubius* plant over the control plant in the study clearly revealed the existence of certain correlations. This is evident from the positive correlation inferred between the trend in the values of total chlorophyll, chlorophyll b, net photosynthetic rate (P_N), stomatal conductance (g_S), intercellular CO_2 concentration (Ci), transpiration rate (E), content of starch, total carbohydrate, fat and protein with the trend in the values of morphological growth parameters obtained in the previous chapter against the concentration of Cr^{VI} stress level. Similarly, the trend in the values of POD, CAT, chlorophyll a content and VPD inferred in the study against the increasing concentration of Cr^{VI} stress level indicates the existence of a negative correlation between the values of these parameters with the level of Cr^{VI} stress as well as with the values of morphological growth parameters. The study concludes that the hexavalent chromium (Cr^{VI}) stress and toxicity is capable of causing disruption and damage to various physiological and biochemical process in *A. dubius* plant in a progressive manner as the concentration of Cr^{VI} stress is increasing and revealed a dose dependent effect. The study further shows the existence of some correlation between the physiological as well as biochemical parameters with morphological growth performances of *A. dubius*, as evident from the inference obtained in the present and previous chapters. On the above

ground, the study suggests these changes and correlation of parameters may be useful as indicators to identify Cr^{VI} stress in *A. dubius* in an area in the future.

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CHAPTER-4

IMPACT OF HEXAVALENT CHROMIUM (Cr^{VI}) STRESS ON ANATOMICAL PARAMETERS

INTRODUCTION

The soil when gets polluted with excess concentration of heavy metals under enormous increase in industrialization together with improper waste management and disposal, the bioaccumulation of harmful toxins occurs which has an adverse impact on the plant anatomy leading to retarded growth process and biomass production (Zhang *et al.,* 2009; Rahoui *et al.,* 2010; Sridhar *et al*., 2011). The oxidative damage under heavy metal stress is found to occur when the generation of reactive oxygen species (ROS) exceeds the scavenging capacity of antioxidants. Depending on the characteristics of ROS like redox potential, chemical reactivity, mobility within the cellular system, they interact with macromolecules such as proteins, enzymes, lipids, pigments, genetic materials and other essential cellular molecules resulting in a set of destructive activities collectively called "oxidative stress" and this is capable of damaging and disturbing the internal structural stability and integrity (Mittler, 2002; Sharma & Dietz, 2006). Several studies have been carried out to identify the effect of oxidative stress induced by heavy metals on structural and ultrastructural changes in different organs of plants.

There are reports that heavy metals such as chromium may cause total destruction of cortical tissue in *Triticum aestivum* seedlings roots (Hasnain & Sabri, 1997). Bianchi *et al*. (1998) reported that when exposed to chromium of 20 and 40 mg l^{-1}, the structural alterations such as disorganization of cells in central cylinder, heavy thickening of tangential walls etc. get induced in the roots, while the impairment noticed in the leaves include increase in intercellular spaces, loss of tissue organization, decrease in chloroplast and starch granule development, change in shape of palisade layer cells and reduction in mesophyll layer of *Mentha aquatica*. Su *et al*. (2005) observed that Cr stress has led to a reduction in the number of palisade and spongy parenchyma cells in the fronds of *Pteris vittata*. Reports indicate that Cr stress causes increased relative

proportion of pith and cortical tissue layers in plants (Suseela *et al*., 2002). It is reported a reduction in the number of spongy parenchyma cells and palisade in the leaves of *Brassica juncea* when subjected to a stress of heavy metals like chromium, caused enhancement in the number of vacuoles and electron dense materials along the walls of xylem and phloem elements, induced clotted depositions in the vascular bundles of stems and roots (Han *et al*., 2004). In this study, considering the above information, an attempt is made to analyses the effect of different concentrations of hexavalent chromium (Cr^{VI}) on the anatomical parameters and growth of *Amaranthus dubius*.

Shanker (2005) has reported that at higher concentrations of chromium, ROS production increases causing oxidative impairment at cellular level and identified that the damage impact is more in the internal root structure compared to shoot, may be because of lesser translocation of Cr from root to shoot and majority of the metal getting concealed in the root vacuoles (Shanker, 2004). Chromium stress develops structural and ultrastructural variations in plant organs and in *Vigna radiata*, changes are observed in the epidermis, cortex, and stele in its stem (Ratheesh *et al*., 2010). It was found that the number of both palisade and spongy parenchyma cells decreased in fronds of *Pteris vittata* at 500 mg/kg of Cr^{VI} concentration and 1000 mg/kg of Cr^{III} concentration (Su *et al*., 2005). An increment in pith and cortical tissue layer proportion has been identified in the roots of *Scirpus lacustris* L. when subjected to excess concentrations of Cr (Suseela *et al*., 2002). As the concentration of heavy metal increases, the distinct characteristics of xylem and phloem in the root system of *Cicer arietinum* are observed to be distorted slowly (Medda & Mondal, 2017). The notable structural variation observed in the roots of Cr-treated *Triticum aestivum* plants has been the collapse of epidermal cells and remarkable increase in cortex thickness (Akcin *et al*., 2018). On observing the light micrographs of Cr-treated roots, it is understood that the breakdown of cortex and endodermal cells occurred in comparison to the control group (Sridhar *et al*., 2011). Cr is identified to be a powerful oxidizing agent which causes acute damage to cell membranes (Barcelo *et al*., 1986; Vazquez *et al*., 1987; Mei *et al*., 2002). Low cellular distinction and thickening of vascular bundles of bush bean stems has been noted by Barcelo *et al*. (1988) due to the accumulation of metals in higher levels. As a result of the bioaccumulation of metal, structural and ultrastructural changes in plant cells are caused, resulting in alteration of plant metabolism and development (Sridhar *et al*., 2011).

When the industrial effluents are less in concentration, there has been a considerable increase in the number of vascular bundles and the response to higher toxicity has been significantly reduced in both the stem and root components of leafy vegetables as reported by Ogunkunle *et al*. (2013). Khan *et al*. (1984), Mahmood *et al*. (2005), and by

Tyagi *et al*. (2012) identified that the width of vessels has been decreased in those plants which are irrigated using effluents from industries. Due to the paramount toxicity of pharmaceutical effluents, a complete collapse or disappearance of structural integrity is found in the phloem cells of roots and stems of plants (Ogunkunle *et al*., 2013). Vazquez *et al.* (1987) has reported the thickening of cell walls and reduced lumen size of xylem and phloem vessels in Cr gathered bush bean plants and the same observation is recorded by Han *et al*. (2004) in mustard plants. Gradual distortion in the characteristic features of xylem and phloem in both root and stem is recorded in *Cicer arietinum* with increased levels of heavy metal Cr and the reason for this may be attributed to enhanced ROS generation causing oxidative impairment at cellular level (Shanker *et al*., 2005). The deformation in the structure caused by metal toxicity is more prominent in root than shoot, probably owing to poor translocation of Cr from root to shoot and majority of the metals get concealed in root vacuoles (Shanker *et al*., 2004).

The intense oxidative activity of Cr^{VI} is seen to cause structural changes in the leaves of Cr treated plants such as palisade and spongy parenchyma cells, shrinkage of epidermal and decrease in intercellular spaces etc. in comparison to control plants (Sridhar *et al*., 2011). A study conducted by Ratheesh *et al*. (2010) on the effect of heavy metal treatments using Cr and Cd on *Vigna* species reported distortion of cortical cell shape in stem and root of *Vigna radiata* to occur; disorganization of root epidermis has been noted in of *V.radiata* due to Cd toxicity; Cr treatment brought about epidermis distortion, cortex and stele in stem of *V. radiata*; whereas the distortion was confined to cortex and stele and lesser in extend in *Vigna unguiculata* and all such distortions in the cells of different tissues may be the result of intervention of heavy metals with cell division or cell elongation. . According to the microscopic studies carried out in brake fern plants by Sridhar *et al.* (2011), cellular breakdown of roots and dehydration and crumbling of internal leaf structure is observed due to Cr accumulation and the Cr-treated plants showed substantiable foliar structural changes in comparison to the control group. Cellular break down adversely affecting the water and elemental uptake by roots in brake fern is observed due to higher concentrations of Cr (Vazquez *et al.*, 1987). Barcelo *et al*. (1986) observed that the roots of bush bean suffered critical injury resulting in water

stress and cellular plasmolysis as a result of Cr toxicity and in brake fern plants accumulation of Cr or Cr-induced water stress or a combination of both resulted in decrease of leaf thickness.

Chloroplast and nuclei structures and membrane ultrastructure of plants is observed to be altered due to the presence of Cr stress (Bassi *et al*., 1990; Mangabeira *et al*., 2011). The studies conducted under electron microscope indicates the damage caused in the membrane systems of plasmalemma, thylakoids, mitochondria, chloroplast envelope and tonoplast in *Spirodela polyrhiza* under Cr stress (Appenroth *et al*., 2003). When exposed to Cr stress at 100 μM for 48 hours, it is noticed that there is deformation in the cell ultrastructure which includes appearance of dense lysosomes like organelles, increased vacuolarization, detachment of the cell wall from the plasma membrane etc. as reported by Panda (2007). Mangabeira *et al*. (2011) identified alterations in the appearance of the nuclei and chloroplasts in *Alternanthera philoxeroides* and *Borreria scabiosoides* when subjected to Cr stress.

Metal toxicity expresses itself in plants in the form of alterations at both structural and ultrastructural levels. Due to direct interactivity of toxic metals with structural constituents at these sites, alterations are induced at cell, tissue and organ levels (Singh & Sinha, 2004). Reduction in the cell size, increase in the number of stomata and trichomes per unit area with simultaneous reduction in the size of the guard cells, more abundant wax coating etc. are some of the variations induced by metal in the leaf epidermis (Weryszko & Hwil, 2005; Azmat *et al*., 2009; Rai *et al*., 2010). In leaves that are treated with heavy metal, the size of guard cells significantly varied as compared to control leaves and it is identified that the size of guard cells in adaxial and abaxial surfaces of leaves decreased with increased level of heavy metal Pb in comparison to control leaves in *Hypericum perforatum* (Ghelich & Zarinkamar, 2013). Those symptoms which are similar to water stress such as increase in the number of stomata, increase in leaf thickness, reduction of the stomata opening, increase in the size of guard cells, palisade mesophyll and size of epidermal cells etc. are observed when subjected to heavy metal treatments (Shi & Cai, 2009). The hypothesis that metals are capable of inducing water

stress is proved with the decrease in stomata aperture size of leaves when subjected to metal toxicity (Singh & Sinha, 2004).

MATERIALS AND METHODS

Transverse sections of stem, root and leaf midrib

Free hand thin transverse sectioning of the stem, root and leaf midrib are made using a sharp razor blade. Plant samples for various anatomical studies are collected from uniform regions of plants for all treatments at uniform growth period. Stem sections are made from region of 4-5cm above the collar region while root sections were made from region of 2-3cm below the collar region. Sections of leaf midrib have been collected from the middle region of the midrib which is collected from leaf occupying the 3^{rd} or 4^{th} position in all treatments. Sections are stained with safranin and mounted on glycerin for microscopic observations and measurements.

a) Root: Diameter of root; length and breadth of epidermal cells
b) Stem: Diameter of stem; length and breadth of epidermal cells
c) Leaf midrib: length and breadth of epidermal cells; length and breadth of cortical cells

Stomatal study

The dried leaf specimens are first rehydrated by soaking in boiled water for about 20 minutes before use. Leaves of the specimens cut at the median portions are soaked in concentrated Trioxonitrate (VI) acid for about 5hours. The appearance of air bubbles indicates the readiness of the epidermises to be separated. The leaf samples are then transferred to Petri dishes containing water and the upper epidermis is separated with the use of fine forceps and dissecting needle (Ibrahim *et al*., 2012). These are then cleaned with camel hair brush in water to remove residual mesophyll layers. The peels are stained with 1% safranin and mounted in glycerol on clean glass slide for microscopic observations and measurements.

The measurements are done on

a) the number of stomata cells per unit area
b) number of epidermal cells per unit area
c) length and breadth of stomata

d) stomatal index (Salisbury, 1927)

$$\text{Stomatal Index (SI\%)} = \frac{\text{No. of stomata per unit area}}{\text{No. of stomata per unit area X No. of epidermal cells per unit area}} \text{ X } 100$$

Photomicrography

Observations, measurements and photomicrographs of the slides of the anatomical sections and epidermal peels are done using Magnus MLX Series Trinocular Microscope fitted with Magnus MIPS-USB Camera (Olympus India).

Statistical Analysis

The mean value and standard deviation of various parameters in the study are determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSIONS

The effect of various concentrations of hexavalent chromium (Cr^{VI}) on anatomical structures of the leaf midrib, stem and root of *Amaranthus dubius* are analyzed. In the leaf midrib, the analysis of epidermal layer and cortical region shows variation in the length and breadth of cells under Cr^{VI} stress (table 4.1). Most of the leaf midrib parameters analysed recorded an insignificant increase over control at lowest stress of 1mg Cr^{VI} treatment while further increase in stress generally recorded a decrease over control, although the decreases are not in a progressive manner along with the increase in Cr^{VI} stress. The variations induced due to 10mg Cr^{VI} stress onwards on the length and breadth parameters of epidermal and cortical cells are generally significantly lower over the control (table 4.2, 4.3, 4.4 & 4.5). Lower-level stress up to 10mg Cr^{VI} and control plants have intact epidermal cells with properly arranged epidermal layer while further increase in the concentration has caused distorted arrangement of epidermal cells along with reduction in size. Below the epidermis, a collenchymatous hypodermal layer is prominent up to 5mg Cr^{VI} treatment but it seems to be absent from 30mg stress treatment onwards. In the cortical region, large round to polygonal shaped properly arranged parenchyma cells with large intercellular spaces have been observed in 1mg Cr^{VI} treated and control plants and in contrast, *A. dubius* grown with further increase in the hexavalent chromium stress has caused the reduction in intercellular spaces along with reduction in cell size and this trend increased as the concentration of treatment increased. Thick walled, curved or wavy appearance of parenchyma cell wall with distorted arrangement is also observed in the cortical regions in higher level concentration treatments while no such observations in control and 1mg stress where parenchyma cells are arranged intact with thin walled and straight margins (figure. 4.1).

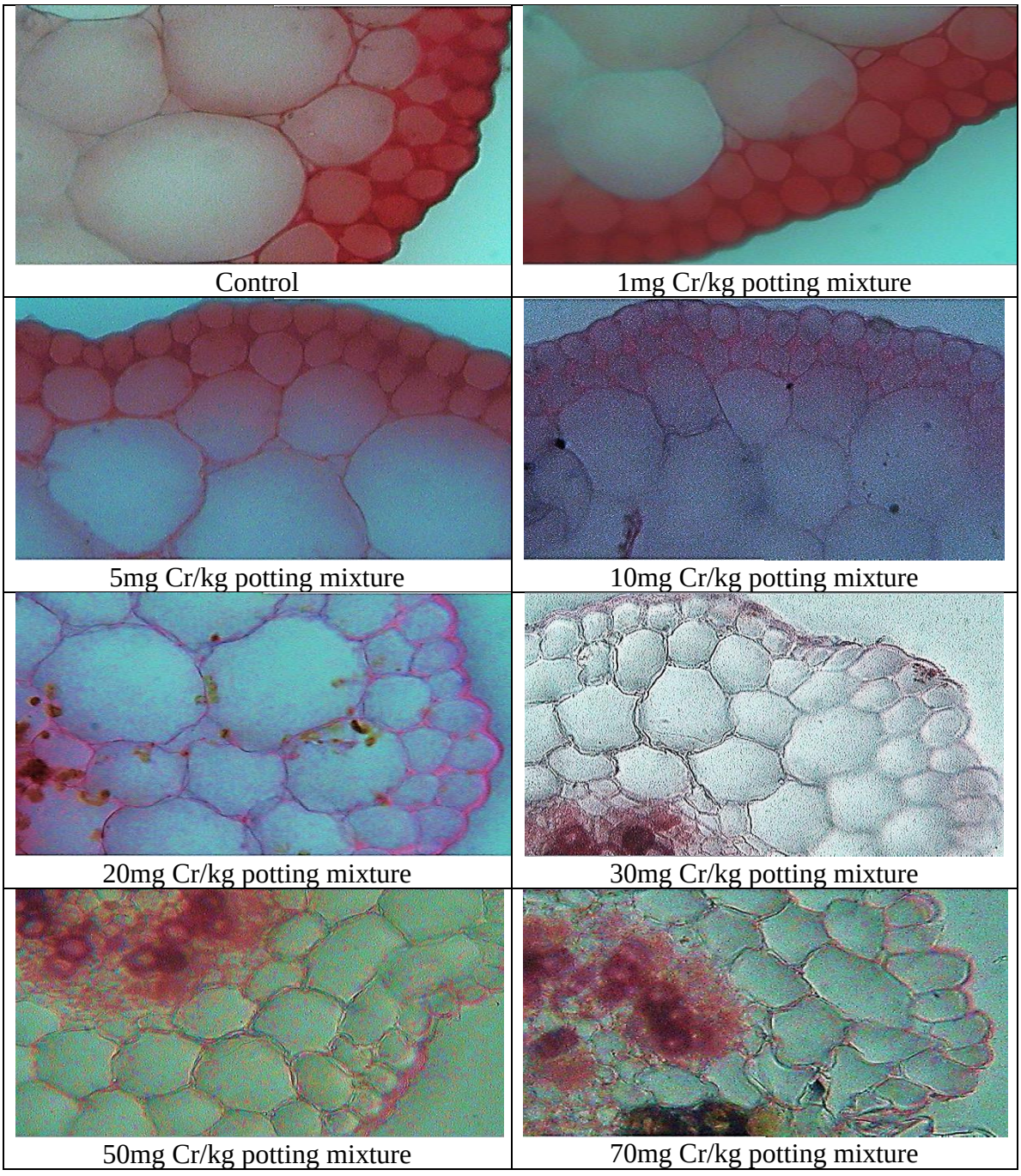


Figure 4.1: Transverse section of leaf midrib in *Amaranthus dubius* plants treated with different concentration stress of Cr^{VI} showing the impact of toxicity

Table 4.1: Impact of Cr^{VI} stress on the cell size of epidermal layer and cortical region in the leaf midrib of *A. dubius*

Treatment	Leaf midrib parameters (in micron)							
Cr^{VI}/mg/Kg soil	Length of Epidermal cells	± SD	Breadth of epidermal cells	± SD	Length of cortical cells	± SD	Breadth of cortical cells	± SD
Control	251.92	23.1	318.33	66.9	1008.7	242.9	1274.1	422.4
1mg	253.83	37.1	392.37	108.0	1113.6	224.7	1290.5	263.9
5mg	257.25	35.2	272.11	63.4	995.56	282.5	1114.6	309.3
10mg	178.59	26.9	245.60	74.7	803.26	220.6	904.76	259.5
20mg	156.63	24.8	182.00	59.6	707.01	348.8	772.32	377.9
30mg	130.19	11.8	192.04	58.3	621.26	128.5	708.70	151.8
50mg	118.86	16.2	198.46	65.9	539.98	220.0	646.98	226.6
70mg	138.58	26.6	215.34	74.2	493.29	144.7	564.46	144.8

Table 4.2: Duncan analysis on the variation in epidermal cell length of leaf midrib in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
7 (50mg)	118.86			
6 (30mg)	130.19	130.19		
8 (70mg)	138.58	138.58		
5 (20mg)		156.63	156.63	
4 (10mg)			178.59	
1 (Control)				251.92
2 (1mg)				253.83
3 (5mg)				257.25
Sig.	.231	.109	.158	.745

Means for groups in homogeneous subsets are displayed.

Table 4.3: Duncan analysis on the variation in epidermal cell breadth of leaf midrib in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
5 (20mg)	182.00		
6 (30mg)	192.04		
7 (50mg)	198.46		
8 (70mg)	215.34		
4 (10mg)	245.60	245.60	
3 (5mg)	272.11	272.11	
1 (Control)		318.33	318.33
2 (1mg)			392.37
Sig.	.066	.110	.086

Table 4.4: Duncan analysis on the variation in cortical cell length of leaf midrib in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	493.29				
7 (50mg)	539.98	539.98			
6 (30mg)	621.26	621.26			
5 (20mg)	707.01	707.01	707.01		
4 (10mg)		803.26	803.26	803.26	
3 (5mg)			995.56	995.56	995.56
1 (Control)				1008.7	1008.7
2 (1mg)					1113.6
Sig.	.160	.084	.051	.162	.421

Means for groups in homogeneous subsets are displayed.

Table 4.5: Duncan analysis on the variation in cortical cell breadth of leaf midrib in *A. dubius* treated with Cr^{VI}

Code	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	564.46		
7 (50mg)	646.98		
6 (30mg)	708.70		
5 (20mg)	772.32	772.32	
4 (10mg)	904.76	904.76	
3 (5mg)		111.46	1114.6
1 (Control)			1274.1
2 (1mg)			1290.5
Sig.	.070	.055	.321

Means for groups in homogeneous subsets are displayed.

The transverse section of stems in *A. dubius* plants treated with different concentration stress of Cr^{VI} shows toxicity induced variation in stem anatomy. With the exception of initial level stress treatment of 1mg Cr^{VI} and in certain cases 1mg and 5mg Cr^{VI} which recorded an insignificant increase over control while all other Cr^{VI} stress treatments have caused reduction in the size of stem diameter and the size of epidermal cells in terms of cell length and breadth over the control. The maximum reduction has been recorded at highest stress of 70mg Cr^{VI} for all the stem parameters (table 4.6). The reductions are significant from 20mg Cr^{VI} treatment onwards with respect to stem diameter whereas with respect to epidermal cell length and breadth, it is significant from 10mg Cr^{VI} treatment onwards (table 4.7, 4.8 & 4.9).

Table 4.6: Impact of Cr^{VI} stress on stem diameter and the cell size of epidermal layer in *A. dubius*

Treatment	Stem parameters (in micron)					
Cr^{VI}/mg/Kg soil	Stem diameter	± SD	Length of Epidermal cells	± SD	Breadth of epidermal cells	± SD
Control	1541.4	15.98	931.55	42.62	328.11	10.00
1mg	1542.6	9.30	932.30	23.95	330.44	10.69
5mg	1532.1	27.11	933.86	31.32	329.49	20.26
10mg	1483.4	62.59	834.08	16.03	177.30	7.39
20mg	1380.0	123.87	714.53	4.33	160.45	5.91
30mg	1244.3	8.36	724.77	12.78	141.60	12.25
50mg	1209.7	6.39	397.39	8.97	124.66	6.54
70mg	983.67	21.06	258.60	48.10	99.86	6.25

Table 4.7: Duncan analysis on the stem diameter variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	983.67			
7 (50mg)		1209.7		
6 (30mg)		1244.3		
5 (20mg)			1380.0	
4 (10mg)				1483.4
3 (5mg)				1532.1
1 (Control)				1541.4
2 (1mg)				1542.6
Sig.	1.000	.248	1.000	.073

Means for groups in homogeneous subsets are displayed.

Table 4.8: Duncan analysis on the variation in stem epidermal cell length in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8	258.60				
7		397.39			
5			714.53		
6			724.77		
4				834.08	
1					931.55
2					932.30
3					933.86
Sig.	1.000	1.000	.528	1.000	.893

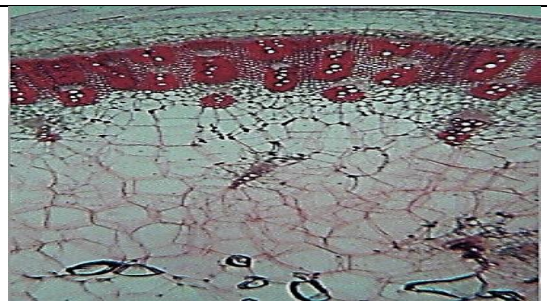
Means for groups in homogeneous subsets are displayed.

Table 9.9: Duncan analysis on the variation in stem epidermal cell breadth in *A. dubius* treated with Cr^{VI}

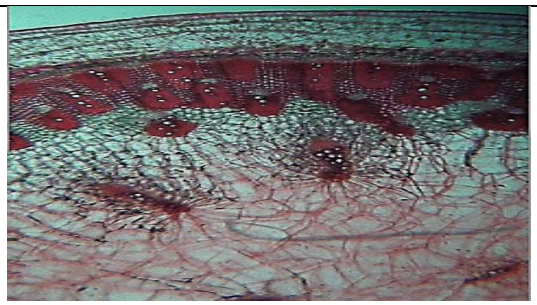
Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
8	99.86					
7		124.66				
6			141.60			
5				160.45		
4					177.30	
1						328.11
3						329.49
2						330.44
Sig.	1.000	1.000	1.000	1.000	1.000	.730

Means for groups in homogeneous subsets are displayed.

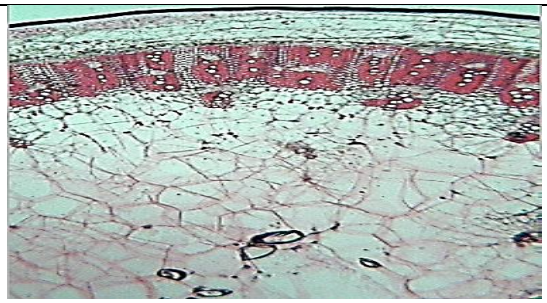
The transverse sections of stem show that, upto 5mg stress treatments of Cr^{VI} had not caused any visible structural changes in the epidermal layer of *A. dubius* compared to control plants where the epidermal cells are larger, normal shaped with intact arrangement. However, from 10mg concentration treatment onwards it is observed that the epidermal cells started flattening and elongating followed by exhibiting irregularity in the cell shape and arrangement. The cells in the hypodermal layer and cortical region seems to be highly disturbed from 30mg concentration treatment onwards and the cells started exhibiting considerable variation in the size, shape and arrangement together with sinuosity in the cell wall margin (figure. 7.3). The analysis of pith cells in the transverse sections of stem in control and 1mg hexavalent chromium treated plants showed large polygonal shaped parenchyma cells having large intercellular spaces with intact arrangement while plants grown in soil treated with 5mg Cr^{VI} onwards started exhibiting irregularity in the cell shape, reduction in cell size and distorted arrangement of cells. The toxic effects are more predominant at 50mg and 70mg concentration treatments. The breakdown of parenchyma tissues is also observed in 70mg Cr^{VI} treatment (figure. 7.4).



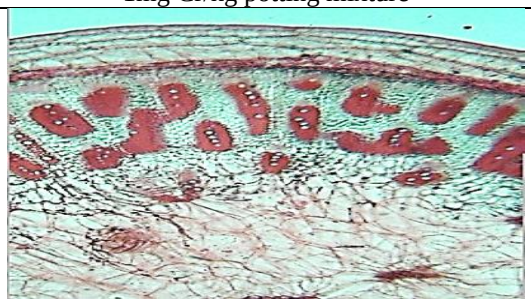
Control



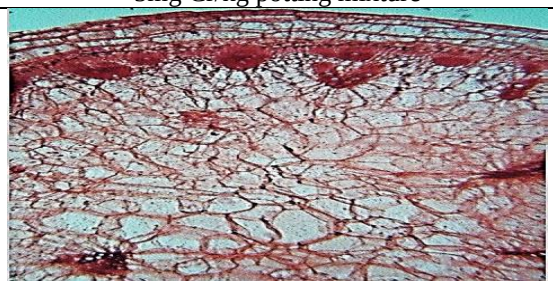
1mg Cr/kg potting mixture



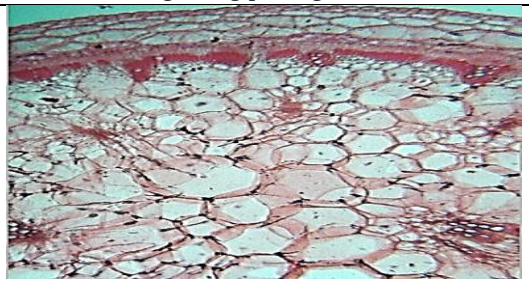
5mg Cr/kg potting mixture



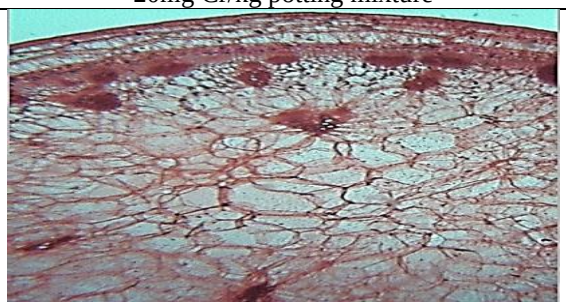
10mg Cr/kg potting mixture



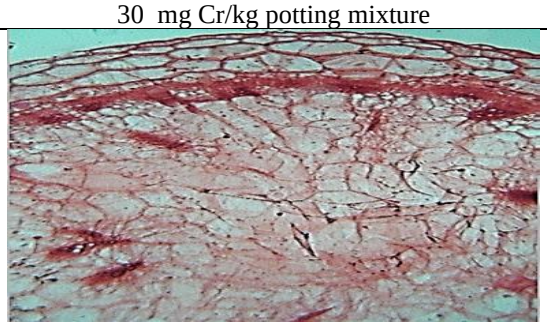
20mg Cr/kg potting mixture



30 mg Cr/kg potting mixture



50mg Cr/kg potting mixture



70mg Cr/kg potting mixture

Figure 4.2: Stem T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) stress showing the impact of Cr^{VI} toxicity induced changes (Magnification: 10x)

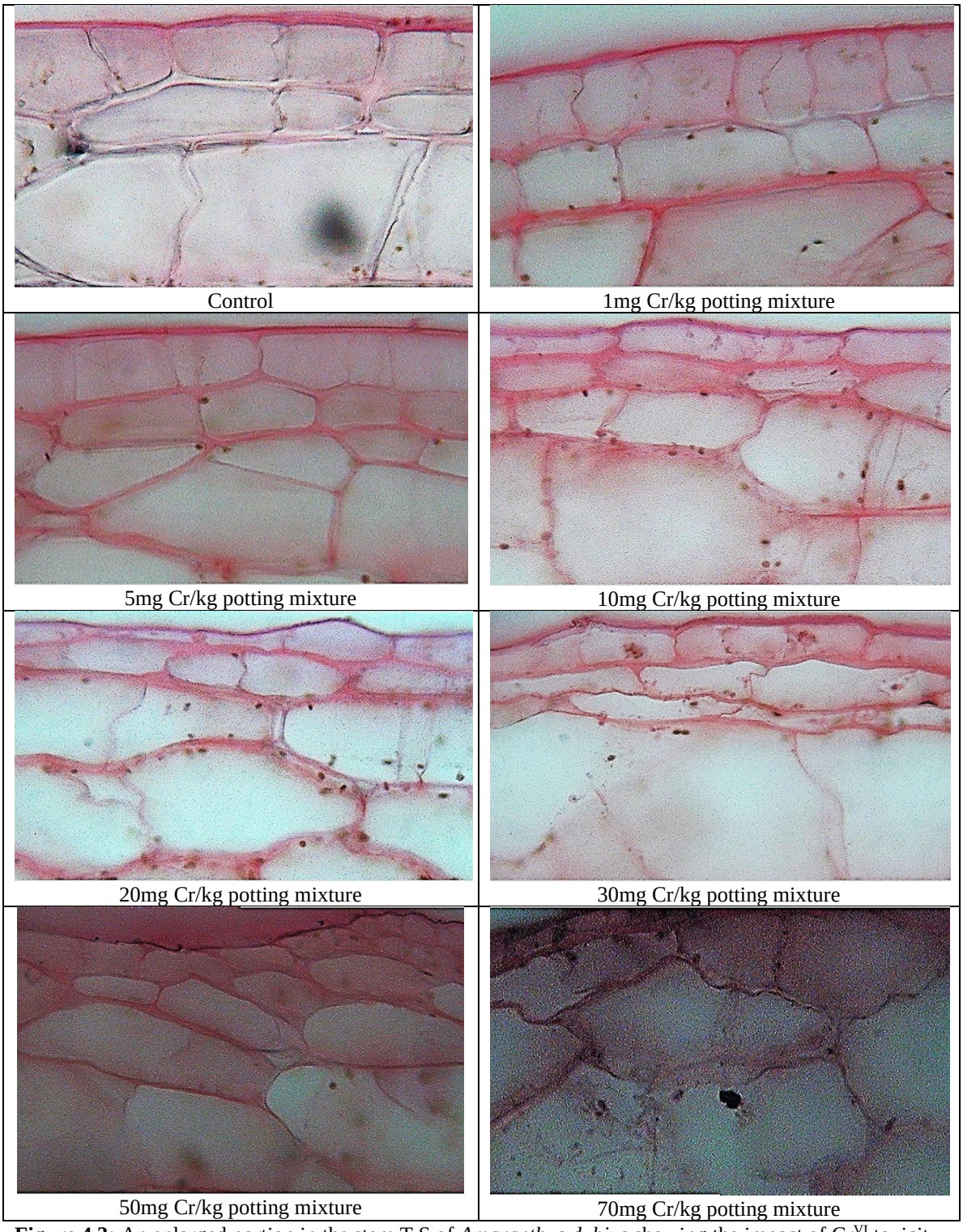


Figure 4.3: An enlarged portion in the stem T.S of *Amaranthus dubius* showing the impact of Cr^{VI} toxicity induced changes in the epidermis, hypodermis and cortex (Magnification: 40x)

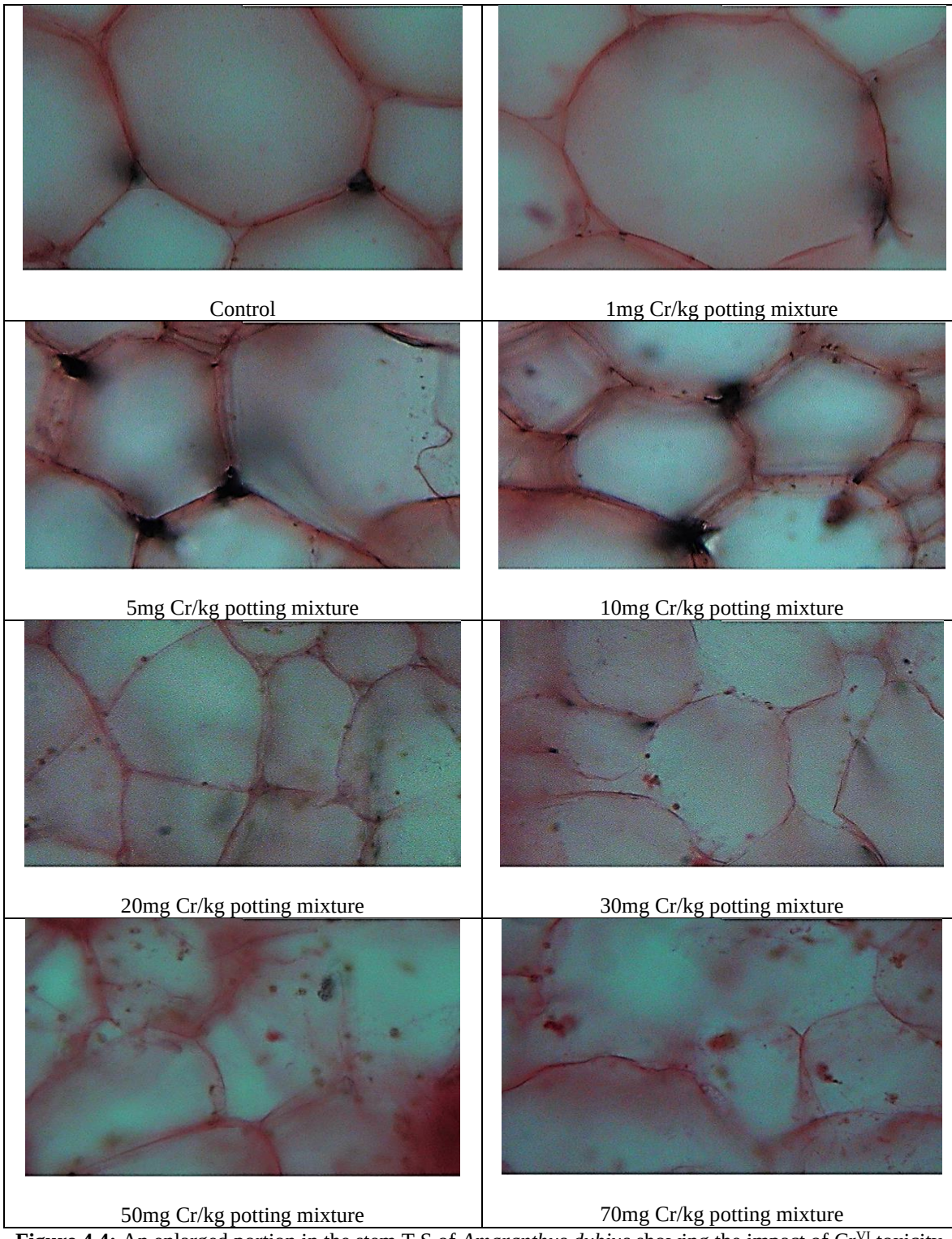


Figure 4.4: An enlarged portion in the stem T.S of *Amaranthus dubius* showing the impact of Cr^{VI} toxicity induced changes in the pith cells (Magnification: 40x)

Stem anatomy also revealed decreasing trend in the number of vascular bundles from 10mg Cr^{VI} concentration treatment onwards and the maximum decrease is observed in the highest stress of 70mg Cr^{VI} concentration treatment (figure. 4.2). The secondary vascular elements form a continuous layer below the pericycle throughout the stem of control and 1mg &5mg Cr^{VI} treated plants and then 10mg onwards the secondary vasculature gradually reduced to patches and finally the maximum reduction is observed in the stem of 70mg Cr^{VI} treated *A. dubius* plants. Vessel elements of vascular bundles in the stems of 1mg Cr^{VI} treated plants are observed to show structural integrity with no observable Cr^{VI} toxicity symptoms when compared to control plants. Similarly, the phloem elements are also found intact with numerous cells. However, from 5mg Cr^{VI} concentration treatment onwards a gradual reduction in the size and number of vessel members and phloem cells are observed which resulted in the loss of structural integrity and collapse of vessel and phloem elements due to hexavalent chromium toxicity and these toxic effects are more prominent in the stem of plants treated with 70mg Cr^{VI} stress (figure. 4.5 & 4.6).

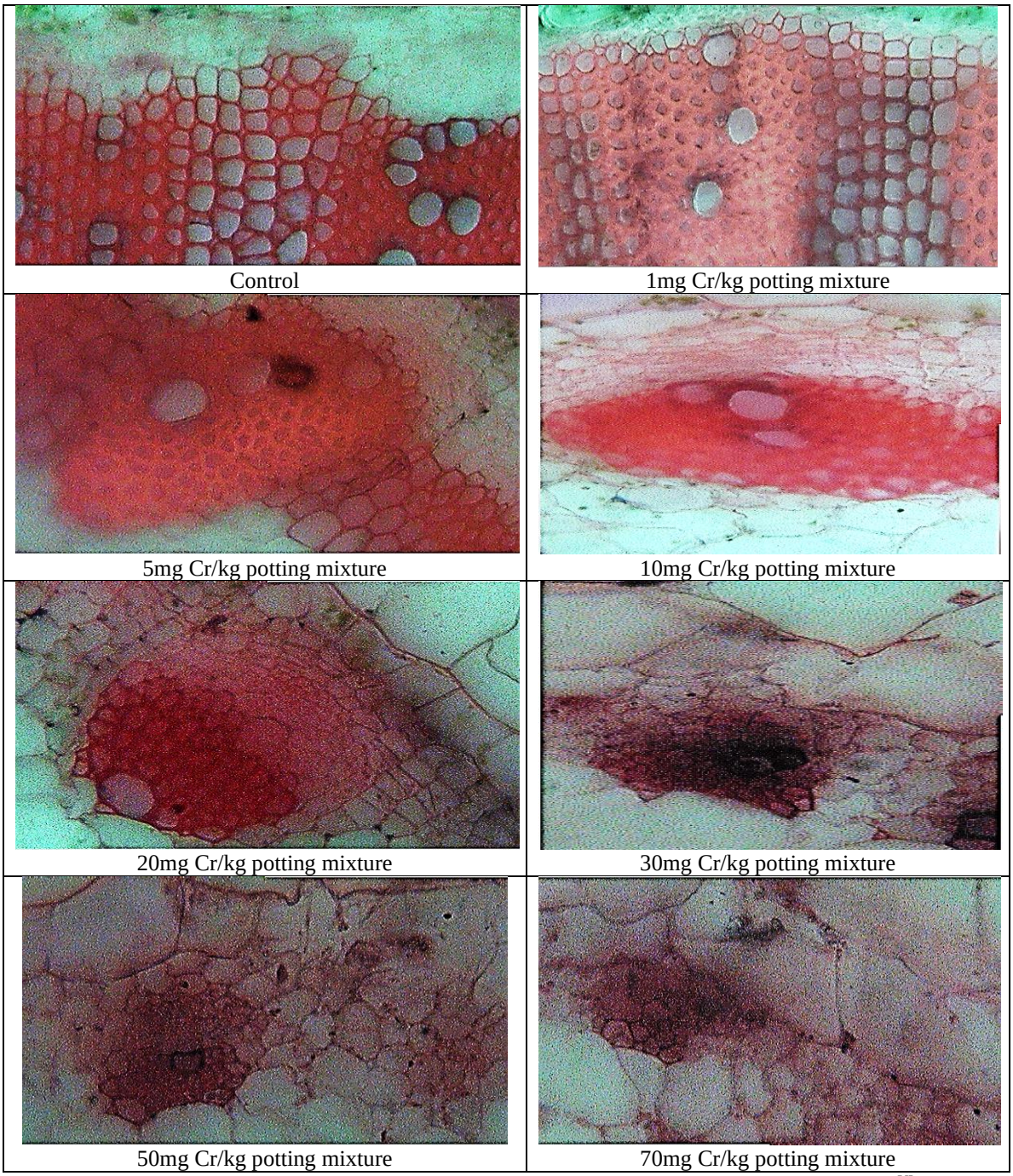


Figure 4.5: An enlarged portion in the stem T.S of *Amaranthus dubius* showing the impact of Cr^{VI} toxicity induced changes in the secondary vasculature (Magnification: 40x)

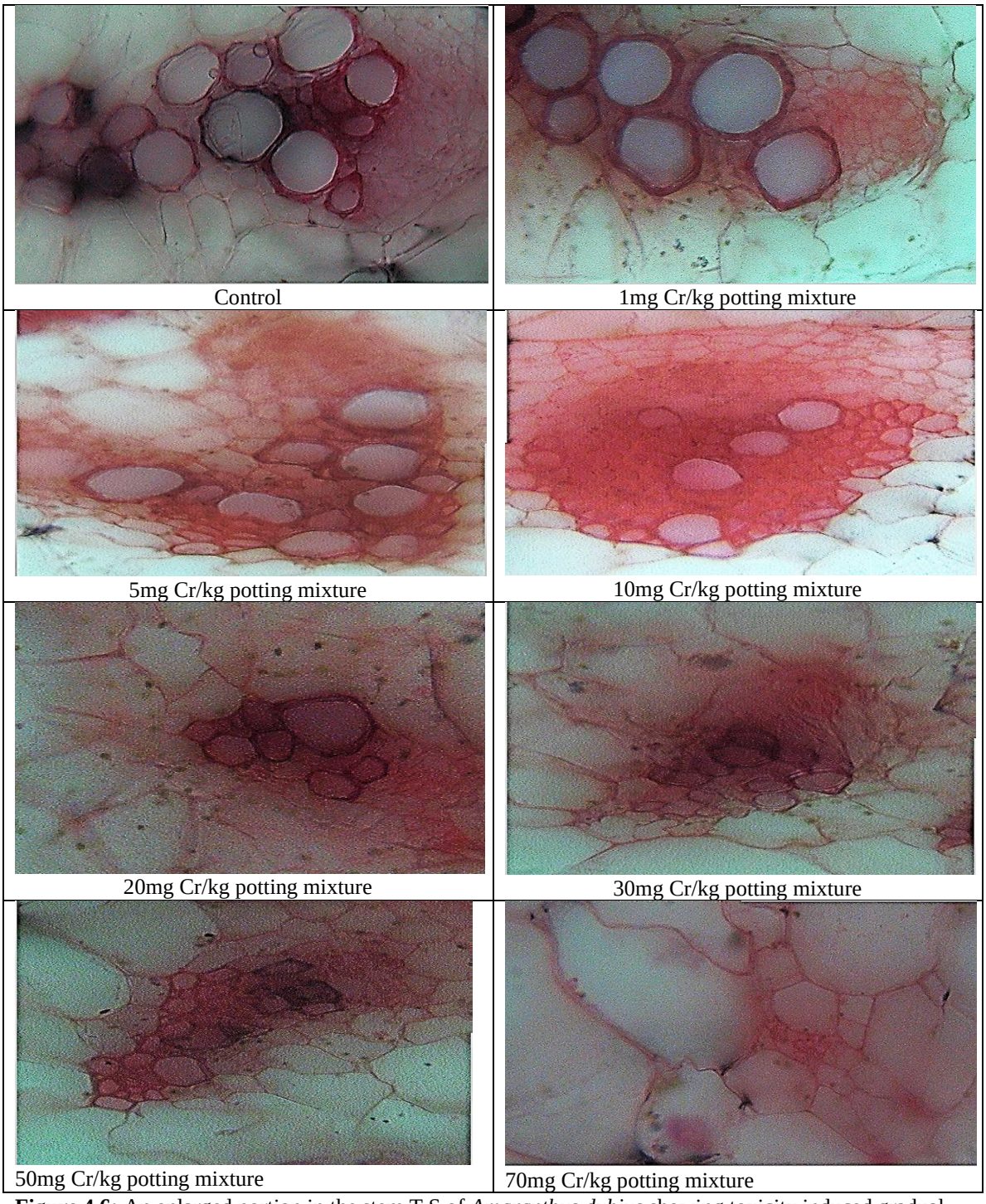


Figure 4.6: An enlarged portion in the stem T.S of *Amaranthus dubius* showing toxicity induced gradual loss of structural integrity and collapse of vessel and phloem elements (Magnification: 40x)

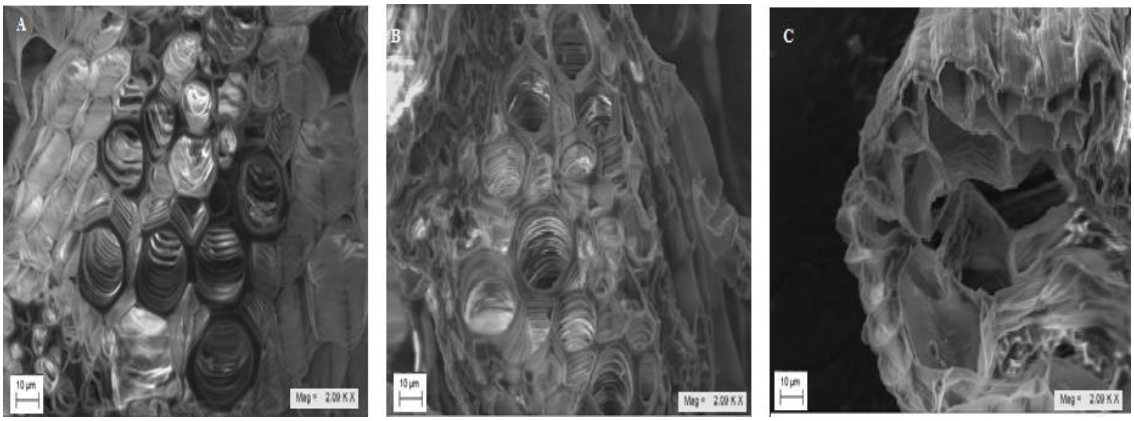


Figure 4.7: SEM micrographs of a portion enlarged in the stem transverse section of *Amaranthus dubius* showing the details of vascular elements (A) Intact arrangement of vessel and phloem elements in control plant (B) loss of structural integrity of vascular elements at 30mg Cr^{VI} stress treated plants and (C) loss of structural integrity and collapse of vessel and phloem elements at 70mg Cr^{VI} stress treated plants

The transverse sections of root in *Amaranthus dubius* plants shows toxicity induced variation in root anatomy. All the Cr^{VI} stress treatments have caused reduction in the size of stem diameter over the control progressively along with increase of Cr^{VI} stress (table 4.10 & figure 4.8**).** With the exception of initial Cr^{VI} stress of 1mg, the reductions in diameter caused by all other Cr^{VI} stress treatments are significant. With respect to the size of epidermal cells, up to 20mg Cr^{VI} stress in the case of cell length and up to 30mg Cr^{VI} stress in the case of cell breadth are only insignificantly influenced, while rest of the Cr^{VI} stress treatments 30mg, 50mg & 70mg for cell length and 50mg & 70mg for cell breadth respectively caused significant increase over control and the maximum increase is recorded at the highest stress 70mg Cr^{VI} in both cases (table 4.11, 4.12 & 4.13).

Table 4.10: Impact of Cr^{VI} stress on root diameter and the cell size of epidermal layer in *A. dubius*

Treatment	Root parameters (in micron)					
Cr^{VI}/mg/Kg soil	Root diameter	± SD	Length of Epidermal cells	± SD	Breadth of epidermal cells	± SD
Control	1238.0	5.13	231.2	18.9	79.60	11.19
1mg	1229.6	5.08	231.2	52.8	79.18	19.09
5mg	1190.8	3.66	223.3	67.9	76.59	14.04
10mg	1112.7	6.75	234.5	39.4	77.72	23.49
20mg	1031.6	11.29	223.5	65.7	77.42	17.06
30mg	908.28	7.33	258.8	38.0	90.98	14.58
50mg	751.57	16.16	307.6	77.3	126.98	24.53
70mg	544.98	13.02	323.0	85.0	135.39	24.94

Table 4.11: Duncan analysis on the root diameter variation in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
8 (70mg)	544.98						
7 (50mg)		751.57					
6 (30mg)			908.28				
5 (20mg)				1031.6			
4 (10mg)					1112.7		
3 (5mg)						1190.8	
2 (1mg)							1229.6
1 (Control)							1238.0
Sig.	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 4.12: Duncan analysis on the root epidermal cell length in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
3	223.28		
5	223.54		
1	231.21	231.21	
2	231.22	231.22	
4	234.52	234.52	
6	258.82	258.82	258.82
7		307.55	307.55
8			321.99
Sig.	.373	.052	.089

Means for groups in homogeneous subsets are displayed.

Table 4.13: Duncan analysis on the root epidermal cell breadth in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05	
	1	2
3 (5mg)	76.59	
5 (20mg)	77.42	
4 (10mg)	77.72	
2 (1mg)	79.19	
1 (Control	79.60	
6 (30mg)	90.98	
7 (50mg)		126.98
8 (70mg)		135.39
Sig.	.265	.454

Means for groups in homogeneous subsets are displayed.

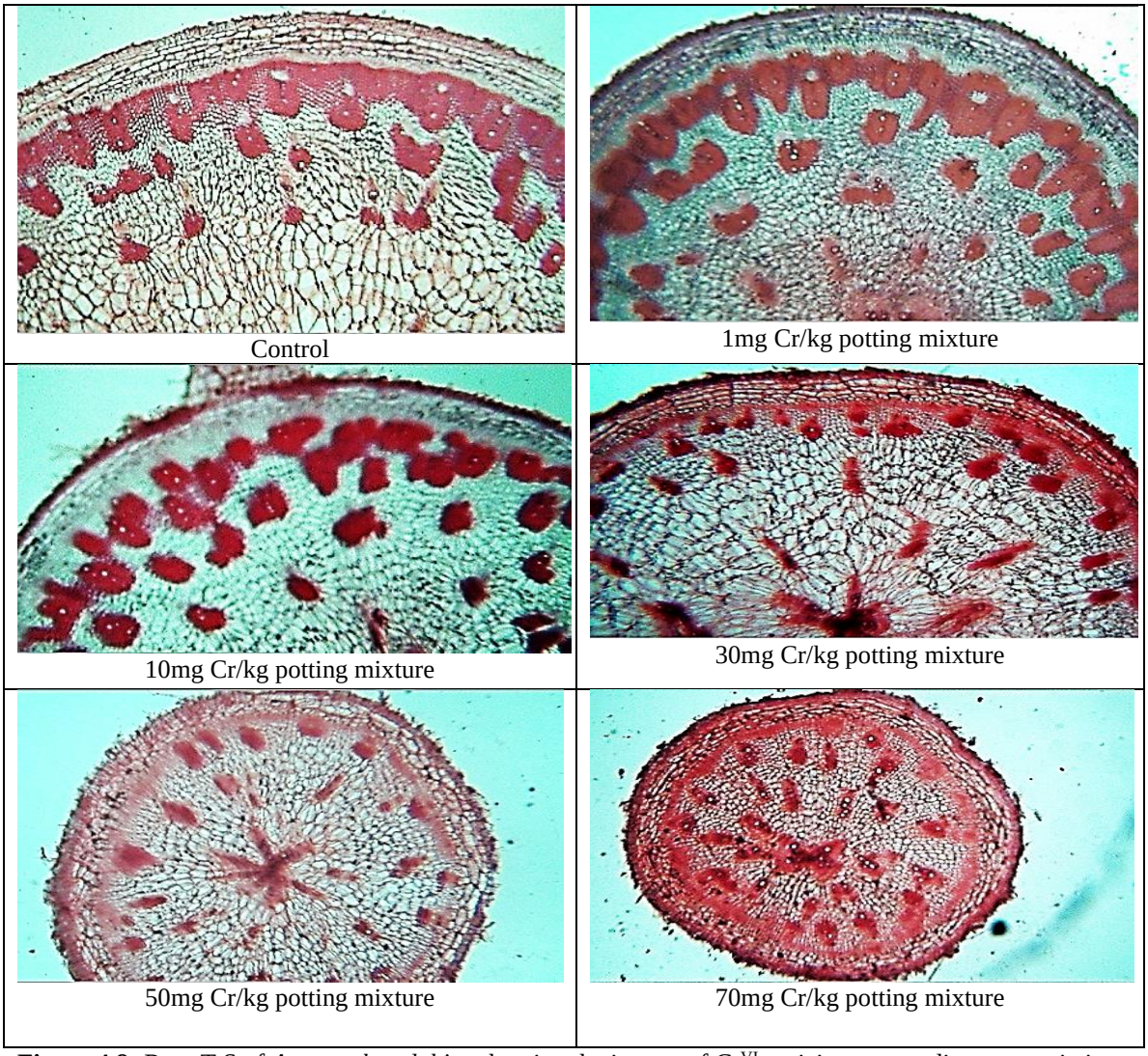


Figure 4.8: Root T.S of *Amaranthus dubius* showing the impact of Cr^{VI} toxicity on root diameter variation and the distribution pattern of vascular bundles (Magnification: 10x)

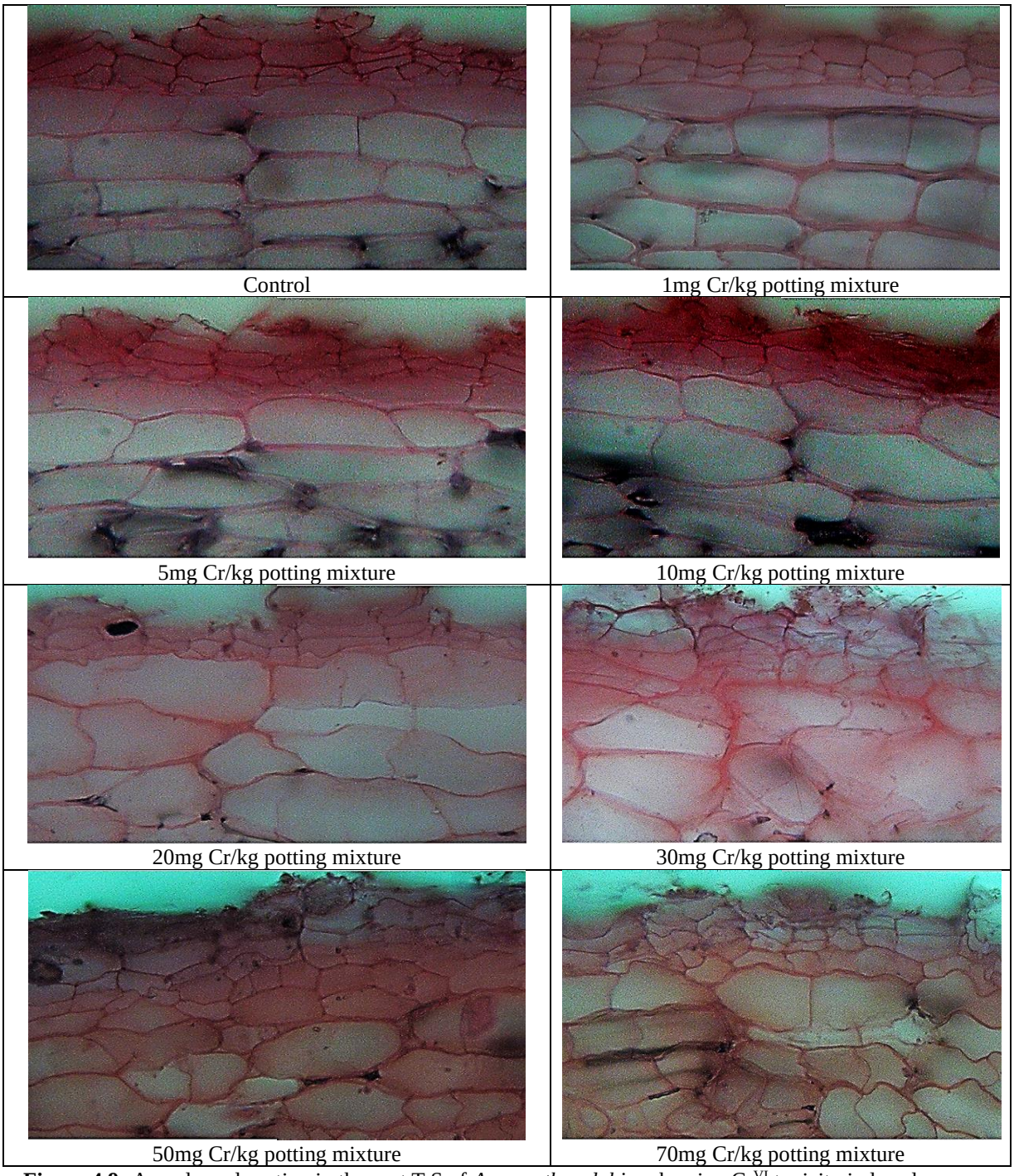


Figure 4.9: An enlarged portion in the root T.S of *Amaranthus dubius* showing Cr^{VI} toxicity induced changes in the epidermis and cortex (Magnification: 40x)

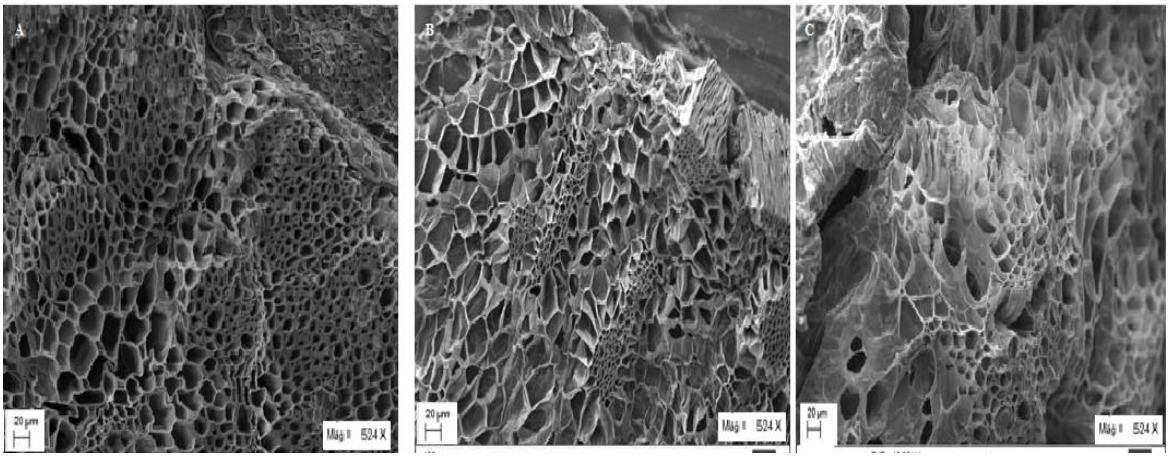


Figure 4.10: SEM micrographs showing the transverse section of *A. dubius* roots. (A) Root of control plant with intact cells (B) Root of 50mg Cr^{VI} stress treated plants lose the intact arrangement and showing irregularity in the cell shape and (C) Root of 70mg Cr^{VI} stress treated plants showing distorted arrangements and breakdown of cells

Almost similar anatomical structural variations induced by hexavalent chromium toxicity in the stem of *A. dubius* are also observed in the transverse sections of root. In control and 1mg Cr^{VI} treated plants, the cells of epidermis and cortical regions are having more or less uniform shape and size with compact arrangement and in contrast, plants treated with 5mg concentration onwards exhibited gradual disturbances and from 20mg concentration onwards cells become highly irregular in size and shape and arranged in distorted manner. The reduction in the development of vasculature especially secondary structural elements has been noticed from 10mg Cr^{VI} stress onwards. Similarly, the cells in the pith region also exhibit toxicity induced irregular shape and size of cells with wavy or curved margin in the cell wall and distorted arrangement and it is more prominent at higher level stress of 50mg and 70mg Cr^{VI} treatments (figure 4.11).

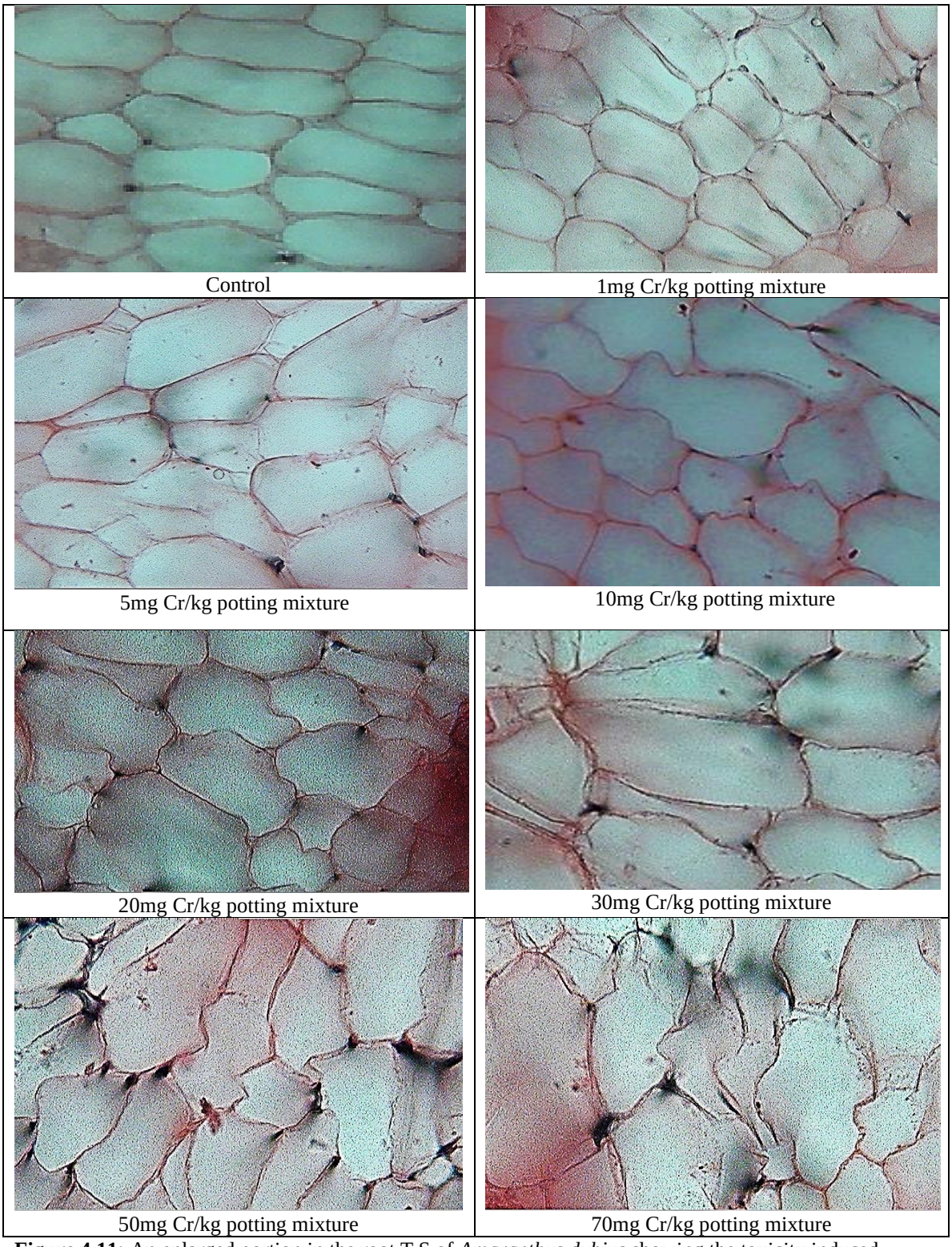


Figure 4.11: An enlarged portion in the root T.S of *Amaranthus dubius* showing the toxicity induced changes in the pith cells (Magnification: 40x)

The changes in the size, irregularity in the shape and distorted arrangements of parenchymatous cells in the epidermal, cortical and pith regions observed in the leaf midrib, stem and root of *Amaranthus dubius*, particularly at higher level stress of 50mg and 70mg/Cr/ kg potting mixture are in similar lines with the observations made by Omosun *et al*. (2009) in *Mucuna* species grown in higher crude oil polluted soil containing heavy metals. Microscopic studies conducted in the brake fern plants by Sridhar *et al*. (2011) revealed that Cr accumulation and its toxicity caused reduction in intercellular spaces and break down of cells compared to the control in brake fern plants. This report is in support of present observation of decreased intercellular spaces, sinuosity in the cell wall margin and cell damages seen in the parenchymatous cells in the cortical and pith region of stems and roots of *A. dubius* at higher Cr^{VI} stress. The reduction in the vasculature in response to metal toxicity observed in the present investigation is also in agreement with the observations and report of Clement *et al*. (2013) that in roots and stems of *Amaranthus hybridus*. They reported reduction in the number of vascular bundles, vessels and phloem elements at lower concentrations followed by total collapse or loss of structural integrity of the vessel and phloem elements under extreme toxicity of the pharmaceutical effluent. The structural changes, distorted arrangements and cellular damages in the root, stem and leaf midrib induced by hexavalent chromium (Cr^{VI}) toxicity in *A. dubius* in the present investigation can be attributed to the intense oxidative action of Cr^{VI} (Sridhar *et al*., 2011). The higher concentration treatments of Cr^{VI} may have led to an increased accumulation of Cr^{VI} in different components, especially in the roots and may result in toxicity induced cellular plasmolysis and water stress affecting water and elemental uptake by the plant. This may be the basic reason for overall decrease in growth performance of *A. dubius* as the concentration of the treatment increased.

The impact of hexavalent chromium stress on stomatal parameters in the adaxial leaf surface of *Amaranthus dubius* is shown in table 4.14. The overall result reveals that the parameters per unit area such as number of stomata cells, number of epidermal cells, total number of cells (stomata + epidermal cells), stomatal index and length & breadth of stomata are more or less negatively influenced by all the Cr^{VI} stress treatments over the control. The reductions in different parameters over control are although not

progressively increasing with increase in Cr^{VI} stress level, generally observed is a gradual decreasing trend towards higher stress levels compared to lower levels (figure 4.12). The statistical analysis reveals that the variations induced by different Cr^{VI} stress levels with respect to stomatal index and breadth of stomata over the control are only insignificant. However, with the exception of initial level stress of 1mg & 5mg Cr^{VI} with respect to the number of stomata; 1mg & 10mg Cr^{VI} with respect to number of epidermal cells and 1mg Cr^{VI} with respect to total number of cells, all other stress treatments caused significant variations over control (table 4.15 – 4.20).

Table 4.14: Impact of Cr^{VI} stress on stomatal index and size of stomata in the adaxial leaf surface of *A. dubius*

Treatment		Stomatal parameters/Unit area				
Cr^{VI}/mg/ Kg soil	No of Stomata cells	No of Epidermal cells	Total No Cells (Stomata + Epidermal cells)	Stomatal index (SI%)	Length of stomata (in micron)	Breadth of stomata (in micron)
Control	6.50±1.7	23.75±1.7	30.25±3.3	0.213±0.03	94.96±12.4	19.75±1.6
1mg	5.50±1.7	22.50±1.9	28.00±2.0	0.197±0.04	76.88±10.8	19.38±4.2
5mg	6.00±1.4	21.00±1.4	27.00±1.2	0.222±0.05	74.55±4.1	18.71±3.7
10mg	4.50±0.6	22.50±1.3	27.00±1.2	0.167±0.02	76.69±4.2	18.8±1.02
20mg	4.50±1.3	18.25±0.5	22.75±1.3	0.196±0.05	68.86±15.6	18.96±4.2
30mg	3.75±0.5	17.50±1.3	21.25±1.7	0.176±0.01	69.68±1.4	18.45±4.5
50mg	4.25±0.5	18.25±1.0	22.50±1.3	0.189±0.02	65.94±1.7	18.41±3.1
70mg	3.25±1.0	16.00±0.8	19.25±1.5	0.167±0.04	63.86±0.8	18.42±0.8

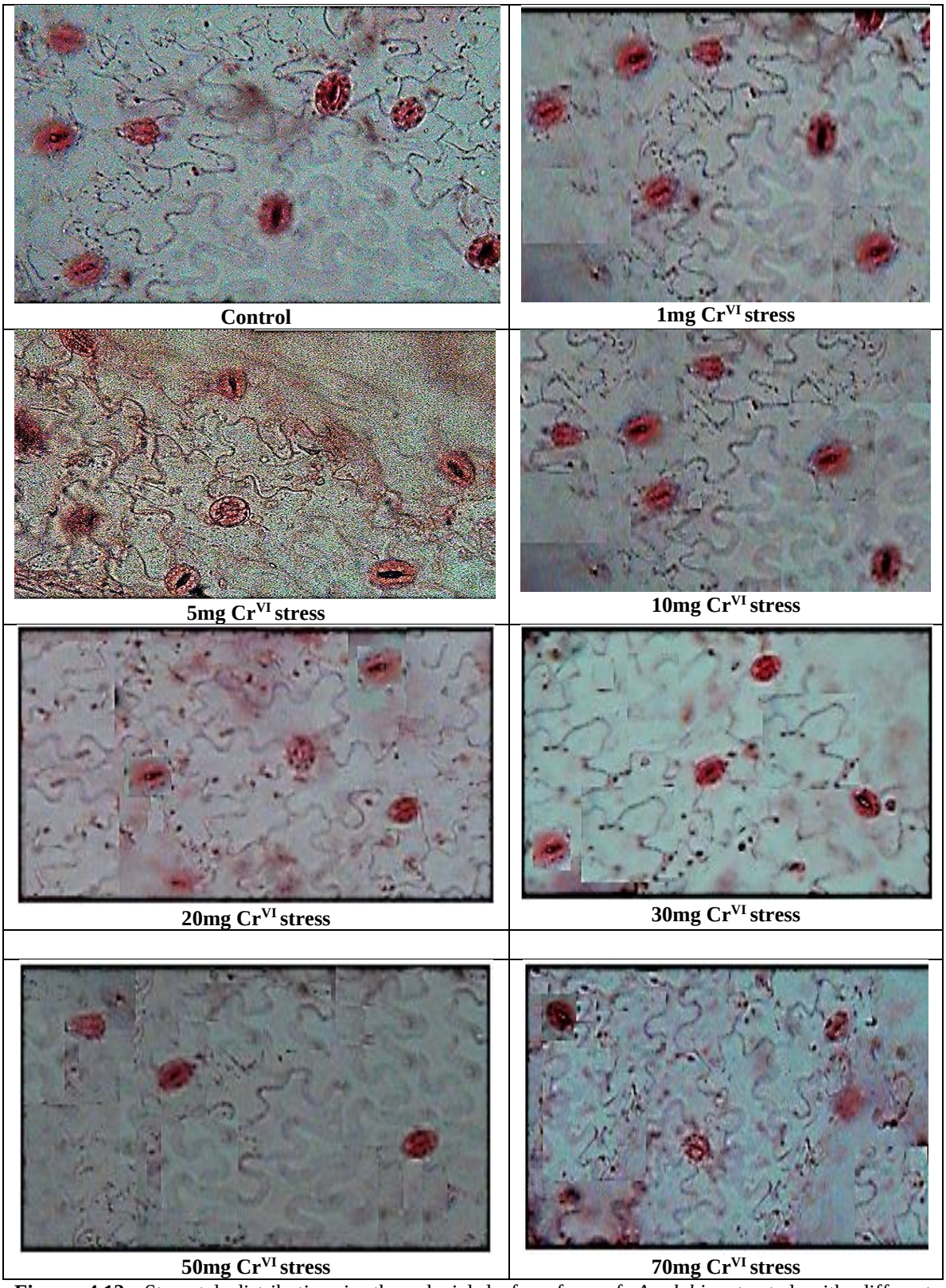


Figure 4.12: Stomatal distribution in the adaxial leaf surface of *A. dubius* treated with different concentrations of Cr^{VI}

Table 4.15: Duncan analysis of number of stomata per unit area in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	3.2500			
6 (30mg)	3.7500	3.7500		
7 (50mg)	4.2500	4.2500	4.2500	
4 (10mg)	4.5000	4.5000	4.5000	
5 (20mg)	4.5000	4.5000	4.5000	
2 (1mg)		5.5000	5.5000	5.5000
3 (5mg)			6.0000	6.0000
1 (Control)				6.5000
Sig.	.157	.050	.050	.230

Means for groups in homogeneous subsets are displayed.

Table 4.16: Duncan analysis of number of epidermal cells per unit area in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	16.0000			
6	17.5000	17.5000		
5		18.2500		
7		18.2500		
3			21.0000	
2			22.5000	22.5000
4			22.5000	22.5000
1				23.7500
Sig.	.119	.453	.138	.215

Means for groups in homogeneous subsets are displayed.

Table 4.17: Duncan analysis of total number of cells (stomata cells + epidermal cells) per unit area in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	19.2500			
6	21.2500	21.2500		
7		22.5000		
5		22.7500		
3			27.0000	
4			27.0000	
2			28.0000	28.0000
1				30.2500
Sig.	.130	.278	.467	.090

Table 4.18: Duncan analysis of stomatal index per unit area in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05
	1
4 (10mg)	0.167
8 (70mg)	0.167
6 (30mg)	0.176
7 (50mg)	0.189
5 (20mg)	0.196
2 (1mg)	0.197
1 (Control)	0.213
3 (5mg)	0.222
Sig.	.059

Means for groups in homogeneous subsets are displayed.

Table 4.19: Duncan analysis of stomatal length in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	63.8617			
7	65.9350			
5	68.8575	68.8575		
6	69.6833	69.6833	69.6833	
3		74.5533	74.5533	
4			76.6900	
2			76.8842	
1				94.9625
Sig.	.122	.117	.055	1.000

Means for groups in homogeneous subsets are displayed

Table 4.20: Duncan analysis of stomatal breadth in *A. dubius* treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05
	1
7 (50mng)	18.4133
8 (70mg)	18.4208
6 (30mg)	18.4517
3 (5mg)	18.7050
4 (10mg)	18.7975
5 (20mg)	18.9600
2 (1mg)	19.3792
1 (Control)	19.7525
Sig.	.395

Means for groups in homogeneous subsets are displayed.

The decrease in size of stomata in terms of stomatal length and breadth recorded in the present study is in similar lines with the reports of Ghelich and Zarinkamar (2013) who observed a reduction in the size of guard cells under the stress of heavy metal Pb compared to the control leaves in *Hypericum perforatum* and also with the reports of Vijayakumar and Udayasoorian (2007), where decrease in stomatal size in the leaves of *Cenchrus cellaris* plants when subjected to stress due to chemical pollutants. There are number of reports on increase in the number of stomata per unit area in plants including vegetables which are grown in stressed environments due to heavy metals (Weryszko & Hwil, 2005; Shi & Cai, 2009; Rai *et al*., 2010) and industrial effluence (Ogunkunle *et al*., 2013). Contradictory to these reports, the present study inferred a decrease in the number of stomata per unit area in all the Cr^{VI} treated *A. dubius* plants over the control. The study further noticed that, although there are significant reductions in the number of stomata recorded particularly at higher level stress of 30mg, 50mg & 70mg Cr^{VI}, the stomatal index variations are not showing any significant reduction over control and this is nothing but due to the corresponding reduction in the number of epidermal cells per unit area. The alterations in the stomatal characteristics such as reduction in the number of stomata and decrease in stomatal size in Cr^{VI} stressed plants over the control inferred in *A. dubius* plants in the study can be considered as modification mechanism as part of survival strategy under water stress and metal toxicity.

CONCLUSION

The reduction in size and number and distorted arrangement of cells, irregularity in cell shape and sinuosity in the cell wall followed by loss of structural integrity and cell damages in leaves, stems and roots and the alteration in the stomatal characteristics such as reduction in number & size of stomata and stomatal index in *Amaranthus dubius* plants treated with hexavalent chromium (Cr^{VI}) stress clearly indicate Cr^{VI} has profound negative impact on anatomy and thereby growth of the plant. With the exception of initial level Cr^{VI} stress of 1mg treatment or 1mg & 5mg treatments in certain cases, all the Cr^{VI} stress treatments have induced more or less visible changes in anatomy over control. Most of the changes in anatomy are observed to increase as the level of Cr^{VI} stress is increased and the changes are more predominant and visible at the highest stress level of 70mg Cr^{VI}/kg soil. Omosun *et al*. (2008) has suggested the use of changes in the anatomy of a plant due to pollution effect as a possible phytoindicator of a polluted environment. The present study suggests that the variety of changes and disturbances in anatomy and the alteration in the stomatal characteristics inferred in *A. dubius* plants in response to Cr^{VI} toxicity may be useful as an indication of Cr^{VI} metal stress in the growing region in future.

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CHAPTER-5

GENO-CYTOTOXIC EFFECTS OF HEXAVALENT CHROMIUM (Cr^{VI}) STRESS

INTRODUCTION

Tremendous developments in the industrial sector of developing countries are taking place with little or no concern for the environmental hazards and its social repercussions (Hussaini, 1997). The mutagenic effluents discharged from the industries and factories into the open environment has an adverse impact on the agricultural sector of most of the countries, especially due to phytotoxicity and bioaccumulation of heavy metals in crops. Ivanova *et al*. (2005) has reported that heavy metals may be classified as the most dangerous and toxic pollutants. The clastogenic and mutagenic impact on the chromosomes caused by ROS production and their functions, induced by heavy metal stress, can disrupt genome stability, gene expression and replication (Britt, 1999; Dovgaliuk *et al*., 2001; Dutta *et al*., 2018). Further, the genomic disturbances induced by the oxidative stress may have an adverse effect on the physiological and biochemical properties in plants eventually leading to retarded growth in plants. Many studies carried out earlier suggest that excessive heavy metal concentration has a significant negative impact on the yield, growth as well as germination of plants (Lakshmi & Sundaramoorthy, 2003; Purohit *et al*., 2003; Ganesh *et al*., 2006; Chidambaram *et al*., 2006; Sundaramoorthy & Ganesh, 2007). The changes caused in the morphological growth due to heavy metal toxicity may be in line with the disruption in cytological and physiological functions in the plant cells which considerably affect respiration, photosynthesis and mitotic activity (Ewais, 1997). When exposed to heavy metals, inhibition in root growth is noticed in plants which may be due to decreased mitotic activity (Hossain *et al*., 2012) and interrupted cell cycle of root meristem (Sundaramoorthy *et al*., 2010). Cytotoxic and genotoxic stress induced in plants due to oxidative toxicity may damage the various cellular components like nucleic acids, membranes and proteins causing typical abiotic stress response (Dutta *et al*., 2018). The first part of the plant that gets exposed to diverse stresses in soil and water, both physical

and chemical, is the root system and hence, the best material identified for cytological and genetical analysis of stress induced changes is the root tip (Odeigah *et al*., 1997). In the light of the above information, the objective of this chapter is to analyze the genotoxic and cytotoxic effect of different concentrations of hexavalent chromium (Cr^{VI}) stress in the root tip cell of *Amaranthus dubius* with the aim of determining the mitotic index and the chromosomal behavioural changes in terms of chromosomal abnormality percentage. This study further aims to relate whether the inferences obtained are correlating with the morphological and physiological growth performances observed in the previous chapters in response to Cr^{VI} stress.

REVIEW OF LITERATURE

Each stage in the life cycle of a plant is influenced by heavy metals. But only unbalanced dosage of heavy metals may cause undesirable genotoxic and cytotoxic impacts leading to genome instability in plant crops (Dutta *et al*., 2018). On exposure to abiotic stress such as heavy metals, reactive oxygen species (ROS) is produced in plant cells which causes oxidative damage to many macromolecules like proteins, lipids, nucleic acids etc. Therefore, ROS induced oxidative stress is a prime cause for mutagenesis, DNA damage and genome instability ultimately hindering plant development and growth thereby dropping crop productivity (Dutta *et al*., 2018). Reduced photosynthetic yield and membrane stability, imbalance in the production of hormones and nutrients, curtailed pigment production, interruption in cell division and gene expression, hindering of DNA replication are the adverse results identified at the biochemical and physiological levels, caused by the oxidative destruction of photosynthetic apparatus and macromolecules which in turn is the resultant of ROS production induced by heavy metals (Singh *et al*., 2009).

Heavy metal stress in plants cause inhibition of root growth and germination of seeds (Chakravarty & Srivastava, 1992; Liu *et al*., 1992) and bring about aberrations in chromosomes and formation of micronucleus (Zhang & Xiao, 1998).). All over the world, natural resources like water and soil are being polluted with genotoxic compounds (Alam *et al*., 2009, 2010; Tabrez & Ahmad, 2011). Genotoxicity is associated with high concentration of heavy metals and it affects the function and structure of DNA, as identified by various studies (Liu *et al*., 2009; Cambier *et al*., 2010). The constitutional integrity of chromosomes is directly influenced by the oxidative damage caused in the DNA (Roy, 2014; Tuteja *et al*., 2009). There are a number of confirmations which prove a solid correlation between oxidative stress induced by heavy metals and genotoxicity which occurs due to genome instability and DNA damage mediated via ROS (Dutta *et al*., 2018). Elevated levels of chromosomal deformity such as stickiness, bridges, chromosome fragmentation and laggards are observed in Chickpea (*Cicer arietinum*) plants grown in soil contaminated with heavy metals like Pb, Zn, Cd and Cr (Siddiqui, 2015). Excess presence of Cd is seen to generate notable percentage of breaks in DNA

double strand and unstable genome in *Vicia faba* which is in addition to oxidative damage of membrane lipids mediated via ROS (Lin *et al*., 2007).

Plant system is a cheap, effective and sensitive bioassay developed for detecting genotoxicity, mutagenicity and cytotoxicity caused by environmental contaminants and wastewaters (Grant, 1999) and it is the plant roots which are commonly adopted in bioassays being the first structure to be subjected to pollutants in soil as well as water (Odeigah *et al*., 1997). According to Oswal *et al*. (1982), the structural and physiological variations in the chromosomes in the course of cell division is caused by the metabolic imbalance due to mitotic poison, interfering with the status, structure and synthesis of nucleic acids. Many authors have observed the alterations in the nuclear structure and hampering of cell division in the roots (Levan, 1945; Corradi *et al.*, 1991; Villalobos *et al.*, 1993). Abu Ngozi (2012) has reported that, the root tip meristem of *Allium cepa* exhibited drastic reduction in mitotic indices, many types of chromosomal aberrations, micro and multiple nuclei and cytokinetic failures as a result of genotoxic and cytotoxic assays of heavy metal contaminated wastewaters. Kiran & Sahin (2006) observed that there is a reduction in the root development and mitotic index due to the impact of heavy metal cadmium in Lentil and its higher concentration resulted in chromosomal abnormalities which include bridge formation at anaphase and telophase, C-mitosis, micronucleus etc. Singh (2015) investigated the genotoxicity and cytotoxicity of Chromium in *Allium cepa* L. using its different concentrations in different time intervals and noticed a significant reduction in root length and the mitotic index with increase in Cr concentration while aberrations in total chromosome increased considerably with increase in Cr concentration.

Heavy metals, if present in excess concentrations shall be toxic causing retarded mitotic functioning and bring about several types of chromosomal abnormalities (Mukherji *et al*., 1990; Gupta, 1992; Abdel, 1996). Genetic instability resulting in DNA damage occurs in plants due to the presence of heavy metals (Liu *et al*., 1992; Gichner *et al*. 2004; Steinkellner *et al*. 1998). Inhibition on DNA synthesis may cause decreased mitotic activities (Bell *et al*., 1976). According to Nefic *et al*. (2013), inter-chromosomal linkages of sub chromatid strands together with excess production of nucleoproteins and

improper protein-protein interaction causes stickness. Kwankua *et.al*. (2012) suggested that on being exposed to heavy metals, the cells are prevented from entering the phases of cell division causing reduction in mitotic index and due to the primary influence of heavy metals on the mitotic spindle, abnormalities like bridges and laggard chromosome are identified during cell division. The cytotoxic chemicals cause spindle interruption which in turn may lead to disruption of chromosome orientation (Selim *et al*., 1981). The toxic impacts on chromosomes induced by heavy metal Cr at the time of cell division includes anaphase bridges, c-mitosis and chromosome stickiness as reported by Zou *et al*. (2006).

Mohandas & Grant (1972) identified chromosomal aberrations as sure indicators of mutagenic as well as mutagenic activity. The proteins, which are an integral constituent of the chromosomes, when attacked by toxic chemicals cause chromosome stickiness as observed by EI-Sadek (1972). Stickiness may be the result of hinderance in spindle formation (Amer & Ali, 1986). Sticky chromosomes are formed due to irregular folding of chromosome fibres which link the chromatids by sub chromatid bridges (McGill *et al*., 1974). According to Das *et al*. (1968), the failure and inappropriate working of the spindle apparatus may be the reason for diagonal orientation in chromosomes. Sreeranjini and Thoppil (2001) understood that breakage at weak areas on the chromosome surface and inability to reunite after the breaks may be the basis of chromosome fragmentation. Premature chromosome condensation is supposed to be the cause of pulverization of chromosomes (Sakari *et al*., 1981).

Adequate concentration of chromium is essential for humans and animals (Mertz, 1967), but not required for plants (Liu *et al*., 1992a), though some studies show that it is good for plant growth (Zheng *et al.*, 1987). Chromium has been extensively used in many industries which discharge enormous amounts of Cr compounds into the atmosphere, in all three forms- solid, liquid and gas- threating the environment biologically and ecologically. Chromium turns out to be toxic in its usual oxidation states of $Cr^{(III)}$ and Cr^{VI} (Bartlett & James, 1979), but Cr^{VI} is identified as the most harmful variant. Abdul Wakeel *et al*. in 2020 has identified the genotoxic, cytotoxic, ultrastructural and photosynthetic changes occurring in plants as a result of the alterations in enzymatic antioxidant system brought about by ROS accumulation induced by chromium stress.

Chromium stress has an impact on the root cells of black gram seedling as reported by Chidambaram *et al*. in 2009.

MATERIALS AND METHODS

Healthy seeds of *Amaranthus dubius* plants collected from Kerala Agricultural University, Thrissur are used in the study. Seeds are soaked in different concentrations of hexavalent chromium (Cr^{VI}) 1mg Cr/litre, 5mg Cr/litre, 10mg Cr/litre, 20mgCr/litre, 30mg Cr/litre, 50mg Cr/litre, 70mg Cr/litre and 0.0mg Cr/litre water (control). Different concentrations of metal chromium are prepared from potassium dichromate salt ($K_2Cr_2O_7$) of analytical reagent grade using distilled water. The seeds are soaked in different treatment solutions for 8 hours. Treated seeds are transferred to germination trays having a size of 2ft x 1ft, provided with a layer of (1.5cm thickness) standard potting mixture (farmyard manure, red soil and sand in the ratio 1:1:1) moistened with corresponding treatment solutions. The preparations are made in triplicates having 50 seeds each and incubated at room temperature of 29± 1°C. After a treatment period of 48 hours for the cytological studies, 20 roots of 1-2 cm long from each treatment group are cut and washed thoroughly in distilled water and immediately fixed in Carnoy's fluid (acetic-alcohol mixture in the ratio 1:2) for 1hour. Roots are then washed in distilled water and treated with 1N HCL for 1-2 minutes. After washing thoroughly in distilled water, the terminal root tips (1-2 mm) are removed and squashed in 2% acetocarmine. The preparations are scanned and photographs are taken by using a Magnus Trinocular microscope fitted with camera (Olympus-Magnus-MIPS-USB).

Cytological studies and calculations are carried out by observing normal and abnormal mitotic phases and interphase cells appearing as per microscopic field during the study. The Mitotic Index (MI) represents the total number of dividing cells in relation to the total number of analysed cells in cell cycle. MI is expressed as percentage of cells undergoing mitosis. Total abnormality is expressed as percentage by counting the total number of abnormal cells in both mitotic and interphase cells out of total number of cells analyzed.

$$\text{Mitotic index (MI)} = \frac{\text{TDC X 100}}{\text{TC}}$$

$$\text{Total percentage of Abnormality } (T_{Abn}) = \frac{TC_{Abn} \text{ X } 100}{TDC}$$

Were,

TDC = Total dividing cells

TC = Total dividing and non-dividing cells

TC_{Abn} = Total count of abnormal cells

Statistical Analysis

The mean value and standard deviation of various parameters in the study are determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSIONS

The results obtained in the study show that the hexavalent chromium (Cr^{VI}) treatment is capable of causing Geno-cytotoxicity and mitotic inhibition in the root tip cell of *Amaranthus dubius* and the level of mitotic inhibition and chromosomal or non-chromosomal abnormalities produced are found to vary with concentration. The effect of different concentrations of hexavalent chromium treatments 1mg, 5mg, 10mg, 20mg, 30mg, 50mg, 70mg/litre and 0.0mg/litre (control) on root tip cells of *A. dubius* obtained in the study is depicted in table 5.1, table 5.2, table 5.3, table 5.4, table 5.5, table 5.6, table 5.7 and table 5.8 respectively.

Although with an exception of misorientation of chromosomes which has been rarely detected, no other abnormalities are observed and recorded in control root tip cells of *A. dubius*. However, the hexavalent chromium (Cr^{VI}) treated root tip cells showed various aberrations which include misorientaton, clumping, stickiness, fragmentation, pulverization, prophase disturbances and interphase disturbances. Different aberrations observed under the misorientation category in the study includes- diagonal or abnormal movement of chromosomes at metaphase and anaphase; the clumping category includes- clumping of anaphase and metaphase; the stickiness includes- stickiness of anaphase and metaphase; the fragmentation/pulverization category includes- fragmentation of anaphase and metaphase and pulverization of anaphase and metaphase; the prophase disturbance includes- disturbed spindle, condensed chromatids and fragmentation of chromatids and finally the interphase disturbance includes- nuclear lesion, binucleate cells, nuclear bulging and elongation, micronuclei, cells without nucleus (ghost cells) and misplaced nucleus.

The present investigation observed that the types of aberration or abnormality and their frequency induced by hexavalent chromium (Cr^{VI}) stress in the root tip cells of *A. dubius* exhibited variations as the concentration of Cr^{VI} varies in the treatment. The kind of aberration induced by Cr^{VI} stress in 1mg concentration is only misorientation of chromosomes at metaphase (1.9%) and anaphase (3.08%) The aberrations induced and recorded due to 5mg Cr^{VI} stress treatment are misorientation, clumping and stickiness and are 2.15%, 2.14% and 0.83% respectively in metaphase stage and 1.97%, 1.75% and

1.16% respectively in anaphase stage of cell division. In 10mg Cr^{VI} stress, maximum diverse types of chromosomal aberrations are detected and recorded in the investigation. Different chromosomal aberrations induced in both metaphase and anaphase stages of cell divisions are misorientation, clumping, stickiness and fragmentation/pulverization (figure 5.1) while disturbances of chromatids and spindle are observed at prophase stage of cell division. The frequency of above-mentioned aberrations is 1.08%, 0.64%, 4.3% and 0.26% respectively at metaphase and 0.82%, 0.9%, 4.02% and 1.09% respectively at anaphase, while at prophase, disturbed chromatids and spindle constitutes around 0.93%. The treatment of root tip cells using 20mg concentration of Cr^{VI} is where the disturbances at interphase stage started (1.78%). In the same time, an overall reduction of 4.11% of chromosomal aberrations at metaphase and anaphase stage of mitosis is recorded compared to responses in 10mg concentration treatment. However, an approximate corresponding increase of 3.76% is recorded in prophase disturbances (figure 5.5). In 30mg concentration of Cr^{VI} stress, more or less similar trend is observed as in 20mg concentration. Here, further reduction in the percent of chromosomal aberrations at metaphase and anaphase is recorded and besides these, the chromosomal aberrations are restricted to stickiness of chromosomes and are 2.16% and 1.89% respectively at metaphase and anaphase stage of cell division (figure 5.6 & 5.7). With respect to prophase and interphase cells, the abnormality percent increased to 7.67% and 11.85% respectively. Disturbed and fragmented chromatids at prophase cells and nuclear lesions at interphase cells are the major disturbance observed. The observations recorded on the abnormalities induced by Cr^{VI} stress at 50mg and 70mg concentration treatments are more or less similar lines. Along with the decreasing trend in the appearance of chromosomal aberrations in metaphase and anaphase as in the previous case, abnormalities related to prophase stage also started a decreasing trend. Metaphase and anaphase aberrations decreased to 1.71% and 1.67% respectively for 50mg concentration treatment and 0.97% and 0.60% respectively for 70mg concentration treatment. Similarly, the frequency of prophase aberrations decreased to 4.38% and 2.79% respectively for 50mg and 70mg concentration treatments. Fragmentation of chromatids, disturbances in spindle, condensation and misplacing of chromatids are the major prophase aberrations observed (figure 5.2). However, the disturbances in interphase cells

recorded a drastic and significant increasing trend and the increase was 29.31% and 51.84% respectively in 50mg and 70mg concentration treatments (figure 5.8). Various abnormalities observed in interphase cells include nuclear lesion, micronuclei, nuclear bulging and elongation, misplaced nuclei and binucleate cells (figure 5.3).

Table 5.1: The chromosomal and non- chromosomal behaviours and mitotic idex in the root tip cells of *Amaranthus dubius* treated as control

Prophase		Metaphase					Anaphase					Telophase		Interphase		Total no of Cells (40x)	Mitotic Index	Total Abnormality
Normal	Disturbances	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Disturbances	Normal	Disturbances			
18.571	0	2.86	0	0	0	0	2.86	0	0	0	0	4.29	0	71.43	0	210	28.57	0
24.615	0	4.62	0	0	0	0	3.08	0	0	0	0	3.08	0	64.62	0	195	35.38	0
18.75	0	2.5	0	0	0	0	3.75	0	0	0	0	1.25	0	73.75	0	240	26.25	0
23.438	0	1.56	1.56	0	0	0	0	0	0	0	0	4.69	0	68.75	0	192	31.25	1.56
13.333	0	2.67	0	0	0	0	4	0	0	0	0	5.33	0	74.67	0	225	25.33	0
19.74 ±4.02	**0**	**2.84 ±1.0**	**0.31± 0.62**	**0**	**0**	**0**	**2.74± 1.43**	**0**	**0**	**0**	**0**	**3.73 ±1.44**	**0**	**70.64 ±3.64**	**0**	**1062**	**29.36 ±4.07**	**0.31± 0.69**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 1062 root tip cells

Table 5.2: Impact of 1mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		**Metaphase**					**Anaphase**					**Telophase**		**Interphase**		**Total no of Cells (40x)**	**Mitotic Index**	**Total Abnormality**
Normal	**Disturbances**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Disturbances**	**Normal**	**Disturbances**			
15.58	0	3.9	2.6	0	0	0	2.6	2.6	0	0	0	3.9	0	68.83	0	231	31.17	5.19
28.05	0	6.1	1.22	0	0	0	3.66	2.44	0	0	0	2.44	0	56.1	0	246	43.9	3.66
12.5	0	4.69	1.56	0	0	0	4.69	6.25	0	0	0	3.13	0	67.19	0	192	32.81	7.81
6.85	0	5.48	2.74	0	0	0	4.11	2.74	0	0	0	1.37	0	76.71	0	219	23.29	5.48
9.72	0	5.56	1.39	0	0	0	4.17	1.39	0	0	0	1.39	0	76.39	0	216	23.61	2.78
14.54± 7.35	**0**	**5.15± 0.77**	**1.9± 0.64**	**0**	**0**	**0**	**3.85± 0.70**	**3.08± 1.65**	**0**	**0**	**0**	**2.45 ±0.99**	**0**	**69.04 ±7.53**	**0**	**1104**	**30.96± 8.42**	**4.98 ±1.93**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 1104 root tip cells

Table 5.3: Impact of 5mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		Metaphase					Anaphase					Telophase		Interphase		Total no of Cells (40x)	Mitotic Index	Total Abnormality
Normal	Disturbances	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Disturbances	Normal	Disturbances			
6.9	0	1.72	3.45	3.45	0	0	3.45	0	1.72	1.72	0	1.72	0	75.86	0	174	24.14	10.34
9.46	0	4.05	0	0	1.35	0	2.7	1.35	4.05	1.35	0	2.7	0	72.97	0	222	27.03	8.11
9.09	0	3.03	4.55	3.03	1.52	0	4.55	3.03	1.52	0	0	0	0	69.7	0	198	30.3	13.64
11.4	0	2.53	1.27	1.27	1.27	0	1.27	2.53	0	1.27	0	2.53	0	74.68	0	237	25.32	7.60
20.6	0	2.94	1.47	2.94	0	0	1.47	2.94	1.47	1.47	0	1.47	0	63.24	0	204	36.76	10.29
11.5 ±4.77	**0**	**2.9 ±0.76**	**2.15± 1.63**	**2.14 ±1.30**	**0.83± 0.68**	**0**	**2.69 ±1.23**	**1.97 ±1.15**	**1.75 ±1.30**	**1.16 ±0.60**	**0**	**1.68 ±0.96**	**0**	**71.29 ±4.53**	**0**	**1035**	**28.71 ±5.06**	**9.99 ±2.38**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 1035 root tip cells

Table 5.4: Impact of 10mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		Metaphase					Anaphase					Telophase		Interphase		Total no of Cells (40x)	Mitotic Index	Total Abnormality
Normal	Disturbances	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Disturbances	Normal	Disturbances			
7.14	1.43	1.43	0	1.43	2.86	0	1.43	0	1.43	4.29	0	0	0	78.57	0	210	21.43	11.43
7.02	1.75	1.75	0	1.75	3.51	0	1.75	0	1.75	3.51	0	1.75	0	75.44	0	171	24.56	12.28
14.67	0	1.33	1.33	0	5.33	0	1.33	1.333	0	4	2.67	1.33	0	66.67	0	225	33.33	14.67
7.35	1.47	0	1.47	0	5.88	0	1.47	1.471	0	4.41	1.47	0	0	75	0	204	25	16.18
10.39	0	1.3	2.60	0	3.9	1.3	0	1.299	1.3	3.9	1.3	1.3	0	71.43	0	231	28.57	15.58
9.31 ±2.96	**0.93 ±0.77**	**1.16 ±0.60**	**1.08 ±0.98**	**0.64 ±0.79**	**4.3 ±1.13**	**0.26 ±0.52**	**1.2 ±0.61**	**0.82 ±0.67**	**0.9 ±0.74**	**4.02 ±0.32**	**1.09 ±1.0**	**0.88 ±0.73**	**0**	**73.42 ±4.07**	**0**	**1041**	**26.58 ±4.54**	**14.03 ±2.08**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 1041 root tip cells

Table 5.5: Impact of 20mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		**Metaphase**					**Anaphase**					**Telophase**		**Interphase**		**Total no of Cells (40x)**	**Mitotic Index**	**Total Abnormality**
Normal	**Disturbances**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Disturbances**	**Normal**	**Disturbances**			
4.92	1.64	0	0	1.64	3.28	0	0	0	0	1.64	0	1.64	0	83.61	1.64	183	14.75	9.84
10	8.57	0	0	0	5.71	1.43	0	0	0	4.29	1.43	0	0	67.14	1.43	210	31.43	22.86
5.36	8.93	0	0	1.79	5.36	0	0	0	0	3.57	1.79	0	0	73.21	0	168	26.79	21.43
6.15	1.54	0	0	1.54	3.08	0	0	0	0	1.54	0	1.54	0	81.54	3.08	195	15.38	10.77
4.17	2.78	0	0	0	4.17	1.39	0	0	0	0	1.39	0	0	83.33	2.78	216	13.89	12.5
6.12 ±2.04	**4.69 ±3.34**	**0**	**0**	**0.99 ±0.86**	**4.32 ±1.06**	**0.56 ±0.69**	**0**	**0**	**0**	**2.21 ±1.54**	**0.92 ±0.77**	**0.64 ±0.78**	**0**	**77.77 ±6.53**	**1.78 ±1.09**	**972**	**20.45 ±8.09**	**15.48 ±6.18**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 972 root tip cells

Table 5.6: Impact of 30mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		Metaphase					Anaphase					Telophase		Interphase		Total no of Cells (40x)	Mitotic Index	Total Abnormality
Normal	Disturbances	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Disturbances	Normal	Disturbances			
5.36	3.57	0	0	0	3.57	0	0	0	0	1.79	0	1.79	0	75	8.93	168	16.07	17.86
1.56	1.56	0	0	0	0	0	0	0	0	3.13	0	0	0	85.94	7.81	192	6.25	12.5
2.9	13	0	0	0	1.45	0	0	0	0	1.45	0	1.45	0	63.77	15.9	207	20.29	31.88
5.48	15.1	0	0	0	4.11	0	0	0	0	1.37	0	0	0	57.53	16.4	219	26.03	36.99
1.69	5.08	0	0	0	1.69	0	0	0	0	1.69	0	0	0	79.66	10.2	177	10.17	18.64
3.40 ±1.72	**7.67 ±5.38**	**0**	**0**	**0**	**2.16 ±1.5**	**0**	**0**	**0**	**0**	**1.89 ±0.64**	**0**	**0.65 ±0.80**	**0**	**72.38 ±10.38**	**11.85 ±3.60**	**963**	**15.76 ±7.87**	**23.57 ±10.35**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 963 root tip cells

Table 5.7: Impact of 50mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		Metaphase					Anaphase					Telophase		Interphase		Total no of Cells (40x)	Mitotic Index	Total Abnormality
Normal	Disturbances	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Misorientation / Diagonal	Clumping	Stickiness	Fragmentation / Pulverization	Normal	Disturbances	Normal	Disturbances			
2.94	4.41	0	0	0	0	0	0	0	0	1.47	0	0	0	73.5	17.65	204	8.82	23.53
3.85	7.69	0	0	0	5.77	0	0	0	0	1.92	0	0	0	46.2	34.62	156	19.23	50.0
1.79	5.36	0	0	0	0	0	0	0	0	3.57	0	0	0	75	14.29	168	10.71	23.21
3.33	1.67	0	0	0	0	0	0	0	0	0	0	0	0	56.7	38.33	180	5	40.0
2.78	2.78	0	0	0	2.78	0	0	0	0	1.39	0	0	0	48.6	41.67	216	9.72	48.61
2.94 ±0.68	**4.38 ±2.09**	**0**	**0**	**0**	**1.71 ±2.30**	**0**	**0**	**0**	**0**	**1.67 ±1.15**	**0**	**0**	**0**	**60.00 ±12.15**	**29.31 ±11.17**	**924**	**10.7 ±5.24**	**37.07 ±13.08**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 924 root tip cells

Table 5.8: Impact of 70mg Cr^{VI} stress treatment on chromosomal and non- chromosomal behaviours and mitotic idex of *Amaranthus dubius*

Prophase		**Metaphase**					**Anaphase**					**Telophase**		**Interphase**		**Total no of Cells (40x)**	**Mitotic Index**	**Total Abnormality**
Normal	**Disturbances**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Misorientation / Diagonal**	**Clumping**	**Stickiness**	**Fragmentation / Pulverization**	**Normal**	**Disturbances**	**Normal**	**Disturbances**			
1.75	1.75	0	0	0	0	0	0	0	0	0	0	0	0	45.61	50.88	171	3.51	52.63
3.03	4.55	0	0	0	1.56	0	0	0	0	1.56	0	1.56	0	48.44	39.06	198	12.5	46.88
1.89	1.89	0	0	0	1.85	0	0	0	0	0	0	0	0	35.19	59.26	159	5.56	62.96
1.45	2.9	0	0	0	0	0	0	0	0	1.43	0	0	0	27.14	67.14	207	5.71	71.43
1.43	2.86	0	0	0	1.43	0	0	0	0	0	0	1.43	0	50	42.86	210	7.14	47.14
1.91 ±0.59	**2.79 ±1.00**	**0**	**0**	**0**	**0.97 ±0.80**	**0**	**0**	**0**	**0**	**0.6 ±0.73**	**0**	**0.6 ±0.73**	**0**	**41.28 ±8.75**	**51.84 ±10.33**	**945**	**6.88 ±3.39**	**56.21 ±10.71**

Each mean data with SD is an average of 15 different microscopic field observations taken from 5 root tips. Data on each parameter is expressed in percent values by observing a total of 945 root tip cells

The results of the present investigation further revealed that the lower-level concentration treatments using 1mg and 5mg Cr^{VI} stress where misorientation of chromosomes at metaphase and anaphase is the most frequent and major abnormality is recorded. This is a total of 4.98% and 4.14% respectively in 1mg and 5mg Cr^{VI} concentration treatments. The diagonal or non-synchronized movement based misorientation of chromosomes at anaphase and metaphase stages of mitosis observed in the study may be due to the genotoxic effect of Cr^{VI} which caused failure or improper functioning of spindle apparatus (Amer & Farah, 1983). In medium level concentration treatments using 10mg and 20mg Cr^{VI}, stickiness of chromosomes is the most frequent and major abnormality recorded and this is a total of 8.32% and 7.1% respectively in 10mg and 20mg concentration treatments. Inter- chromosomal linkages of sub-chromatid strands coupled with excessive formation of nucleoproteins and inappropriate protein-protein interaction might be the reason for stickiness of chromosomes at metaphase and anaphase stage of cell division (Nefic *et al*., 2013). The stickiness of chromosomes can also be attributed to the inhibition of spindle formation due to the interference of Cr^{VI} in the nucleic acid metabolism (Amer & Ali, 1986).

However, in higher level concentration treatments using 30mg, 50mg and 70mg concentration of Cr^{VI} where nuclear disturbances characterized by morphological, structural and positional alterations in the interphase nuclei are the most frequent and major abnormality is recorded. This is 11.9%, 29.31% and 52.02% respectively in 30mg, 50mg and 70mg concentrations. Among the various nuclear disturbances identified in the root tip cells of *A. dubius*, binucleated cell formation may be the result of cytotoxic effect of Cr^{VI} which caused inhibition of cytokinesis following telophase. The nuclear bulging or elongation observed in nuclei of interphase cells constitute an unusual response to hexavalent chromium toxicity and they may be the result of an internal pressure and local loosening of the nuclear envelope resulting in the outward distention (Eleftherios *et al*., 2015).

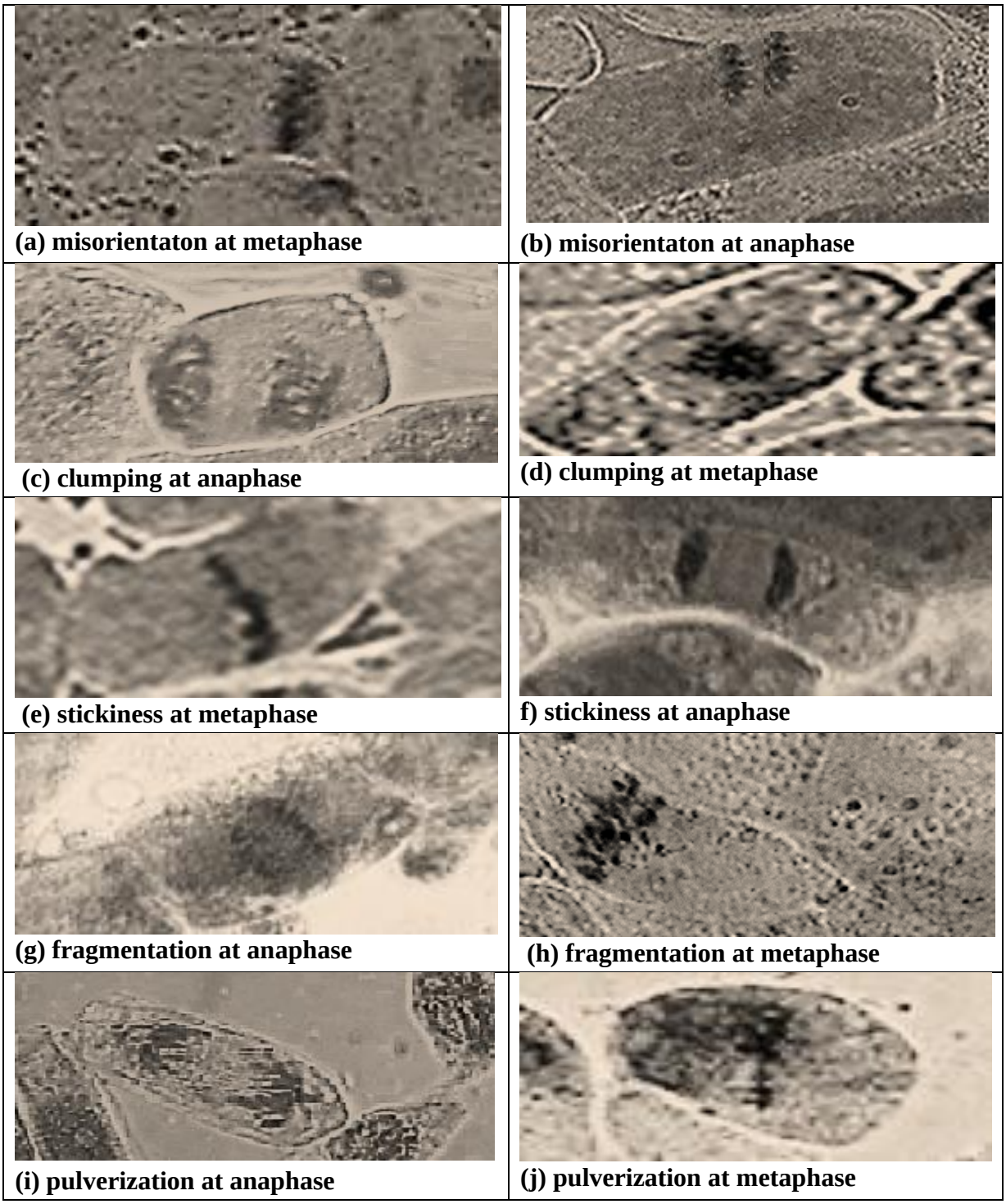

Figure 5.1: Photomicrographs of chromosomal aberrations at Anaphase and Metaphase stage of mitosis induced by different concentrations of hexavalent chromium (Cr^{VI}) in the root tip cells of *Amaranthus dubius* (magnification: 1000x)

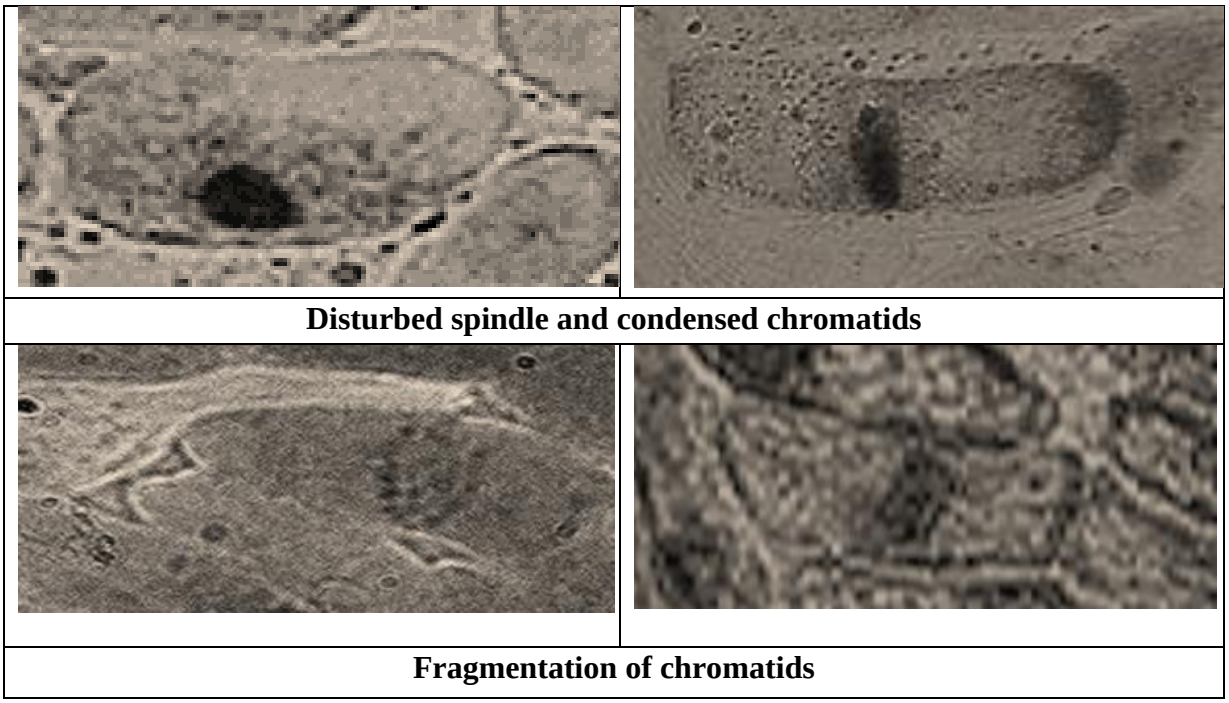

Figure 5.2: Photomicrographs of chromosomal aberrations at Prophase stage of mitosis induced by different concentrations of hexavalent chromium (Cr^{VI}) in the root tip cells of *Amaranthus dubius* (magnification: 1000x)

The appearance of micronuclei (MN) is generally considered as biomarkers of genotoxic events and chromosomal instability and they may be formed as a consequence of chromosome breakage or may be because of whole chromosomes that are not incorporated to the main nucleus during the cell division cycle (Hilada *et al*., 2013; Fenech, 2007; Fenech, 2011). The induction of micronuclei can also be attributed to Cr^{VI} toxicity induced improper functioning of spindle together with defective anaphase checkpoint genes, disruption in the kinetochore protein structure and its assembly which lead to the disturbances in the segregation of whole chromosomes at anaphase. Different nuclear abnormalities observed in interphase cells may also be attributed to severe toxicity which cause damage to nuclear DNA, inhibition of DNA synthesis; toxicity induced improper quantity or quality of histones or other proteins responsible for proper chromatin organization and arrangement (Jin *et al*., 2008). The investigation further observed that at higher level concentration treatment especially at 70mg Cr^{VI}/L, significant reduction of prophase cells accumulation is recorded compared to control. At

the same time, considerable enhancement in the accumulation of interphase cells compared to control is also recorded. These observations indicate that higher concentrations of hexavalent chromium are effective in breaking the normal onset of prophase during mitosis. This clearly reveals that hexavalent chromium interferes with normal sequence of mitosis and has the potentiality to arrest cell division and this might be due to the depolymerization of microtubules and damage to microtubule protein caused by Cr^{VI} toxicity and failure in the organization of microtubules into a bipolar prophase spindle surrounding the nucleus in late interphase cells (Dorota, 2011). It can also be attributed to severe toxicity which cause direct damage to nuclear DNA and block the interphase cells from entering the mitotic cell cycle.

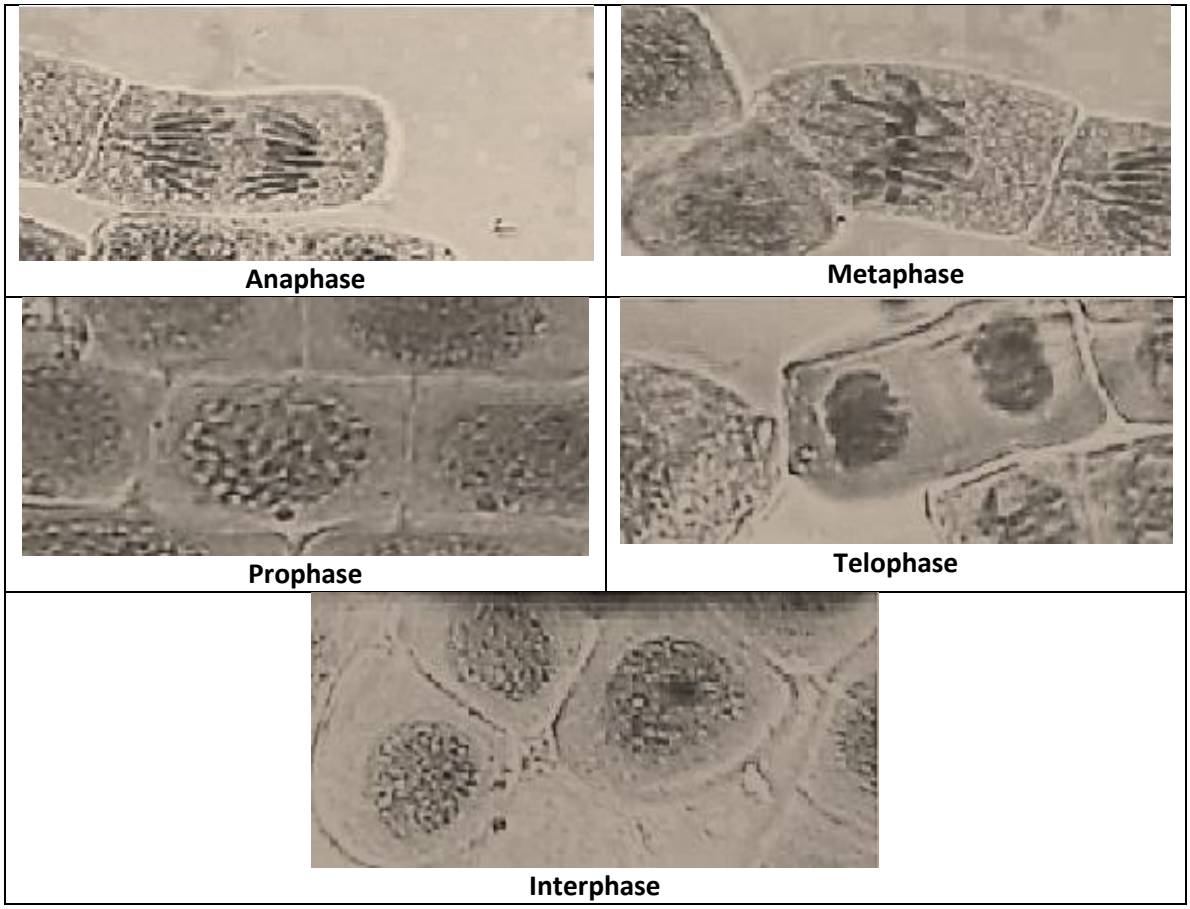

Figure 5.2A: Photomicrographs of normal stages of mitosis in the root tip cells of control treated *Amaranthus dubius* (magnification: 1000x)

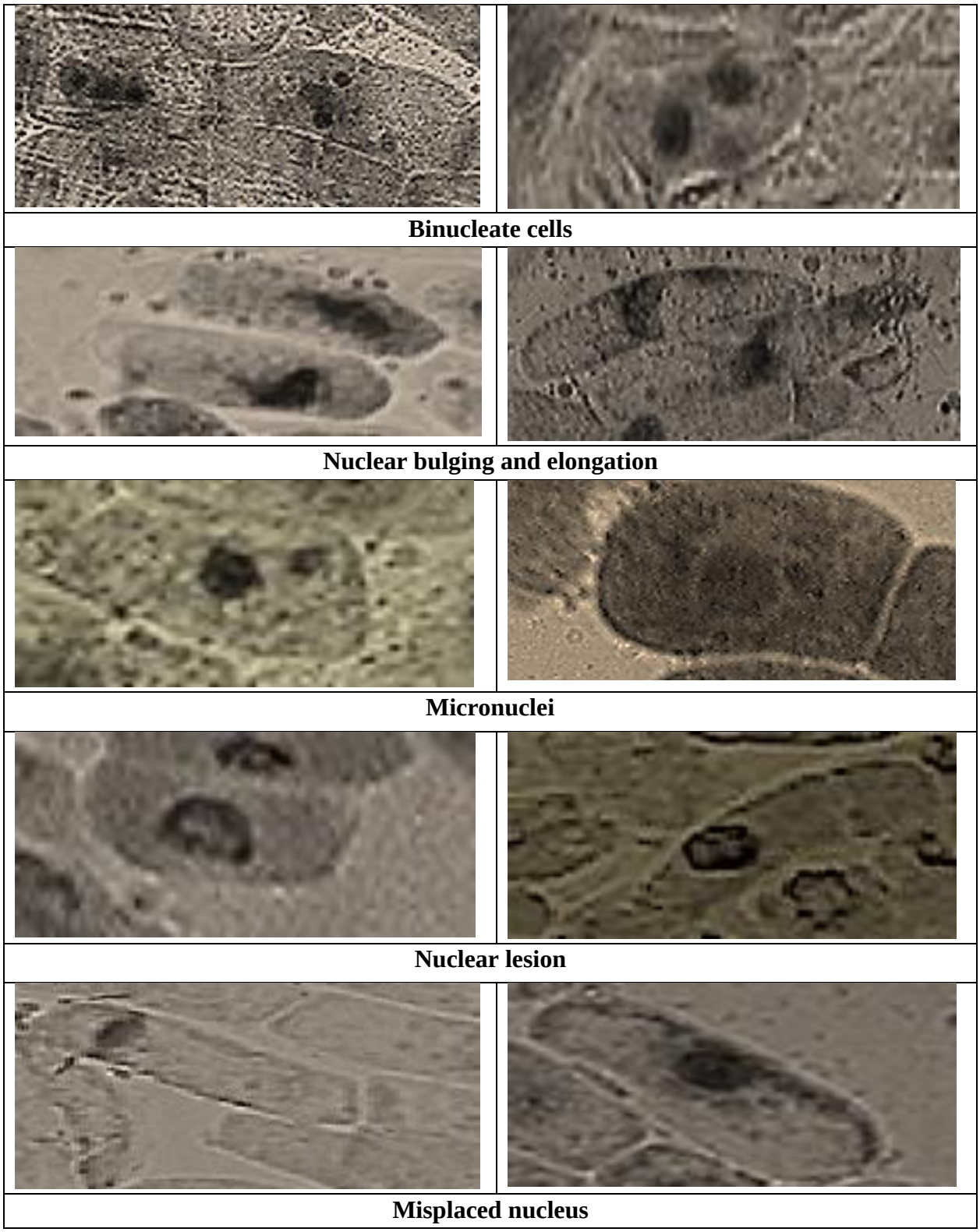

Figure 5.3: Photomicrographs of non-chromosomal abnormalities at interphase stage of mitosis induced by hexavalent chromium (Cr^{VI}) stress in the root tip cells of *Amaranthus dubius* (magnification: 1000x)

Other type of chromosomal aberrations like fragmentation of chromosomes observed in the study during the anaphase and metaphase stage of cell division might be the result of structural instability caused by Cr^{VI} toxicity which led to the breakage at the fragile sites on the chromosome (Sreeranjini & Thoppil, 2001). The pulverization of chromosomes is assumed to be due to the premature condensation of chromosomes (Sakari *et al*., 1981). Cr^{VI} toxicity induced metabolic imbalance which led to improper functioning of spindle and structural disturbances in chromosomes may be the reason for clumping of chromosomes. Fragmentation of chromatids observed in the prophase may probably be due to severe toxic metal-chromatid interaction while disturbed spindle and condensed chromatids may be the result of toxicity induced failure in the organization of microtubules and disruption or damage of microtubule network. Another cytotoxic abnormality induced by Cr^{VI} toxicity in the study is ghost cells (cell without nucleus) - which are dead cells in which the outline is visible but nucleus and cytoplasmic structure is not stainable. Besides ghost cells, alterations in shape and size of cells such as gigantic, curved and extended cells are also observed in the study due to higher level hexavalent chromium toxicity (figure 5.4).

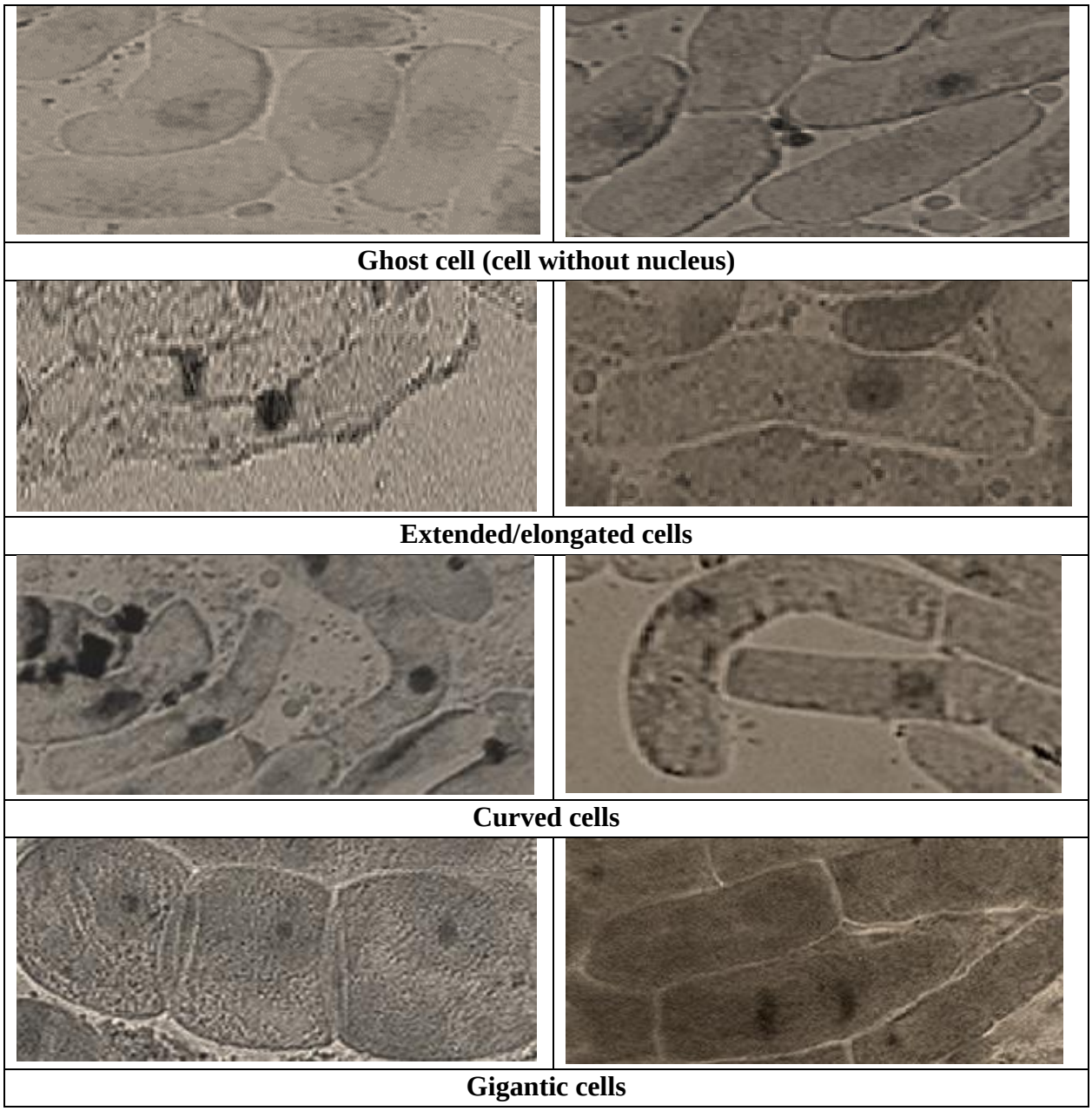

Figure 5.4: Photomicrographs of other cytological abnormalities induced by hexavalent chromium (Cr^{VI}) stress in the root tip cells of *Amaranthus dubius* (magnification: 1000x)

The investigation reveals that during the initial level concentration to a medium level concentration treatment (1mg-20mg) using hexavalent chromium (Cr^{VI}), the chromosomal aberrations are recorded in higher frequencies but considerably reduced at higher levels (30mg-70mg), however the cell division significantly decreased. Chromosomal aberrations provide a sensitive endpoint for assessing the genotoxicity of chemicals (Topashka *et al*., 2003) and the chromosomal aberrations observed in this study point to the genotoxic potential of hexavalent chromium (Cr^{VI}) which probably has effect on the mechanism of cell division in the root tip cell of *A. dubius*. Chromium induced genotoxic effects during cell division observed by Levan (1945) and Liu *et al*. (1992a) in their study are in agreement with the findings of the present investigation. The suppression of mitotic activity is often used to assess cytotoxicity (Smaka-Kincl *et al*., 1996). The higher frequency of cells with disturbances in the interphase at higher concentrations of hexavalent chromium is indicator of the cytotoxic effects of tested heavy metal. The decrease in the mitotic index indicates that Cr^{VI} can arrest cell growth. Cr^{VI} induced alteration of nuclear structure and inhibition of cell division at higher concentration in plant roots has been observed by several authors (Levan, 1945; Corradi *et al*., 1991; Villalobos *et al*., 1993).

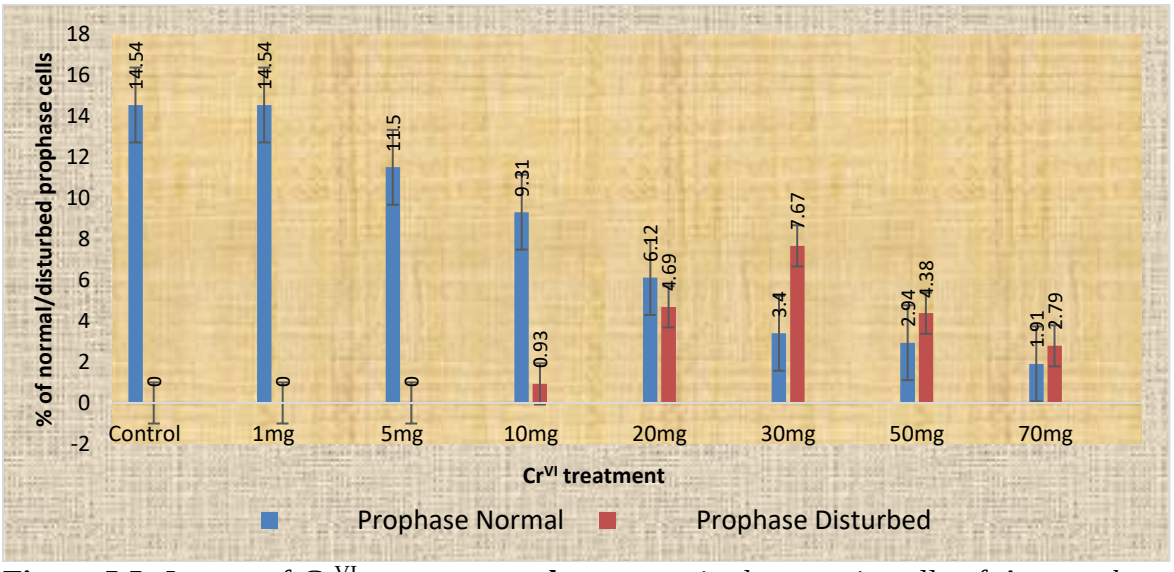

Figure 5.5: Impact of Cr^{VI} stress at **prophase stage** in the root tip cells of *Amaranthus dubius*

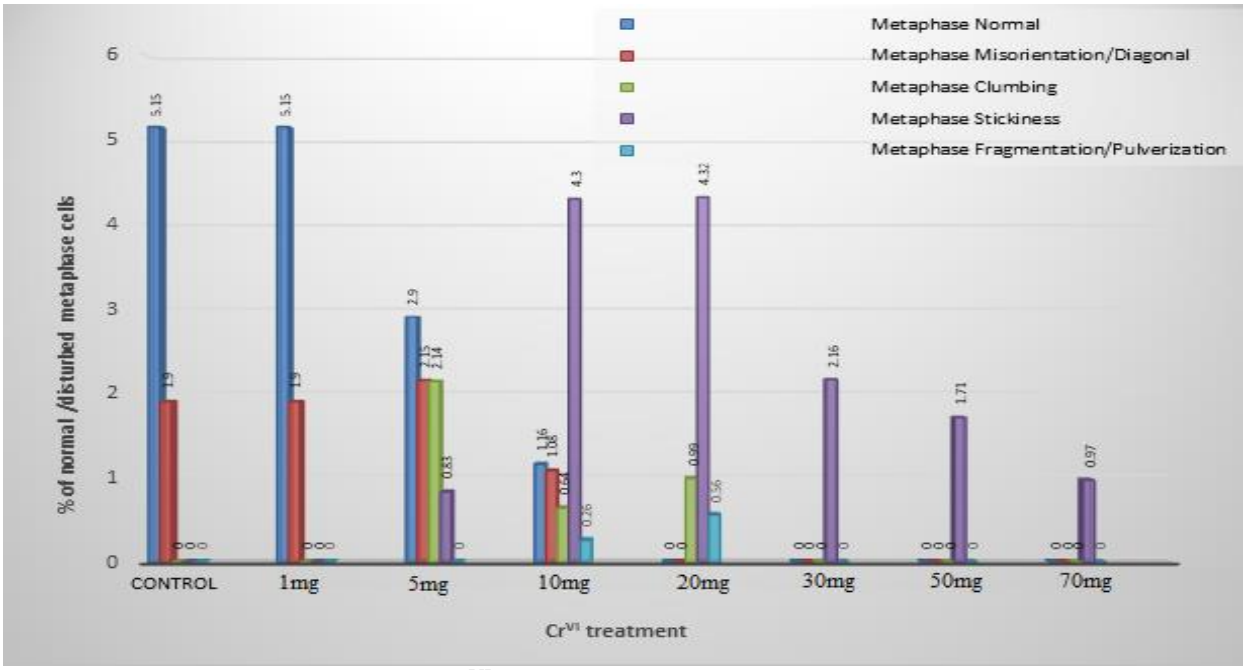

Figure 5.6: Genotoxic effect of Cr^{VI} stress at **metaphase stage** in the root tip cells of *Amaranthus dubius*

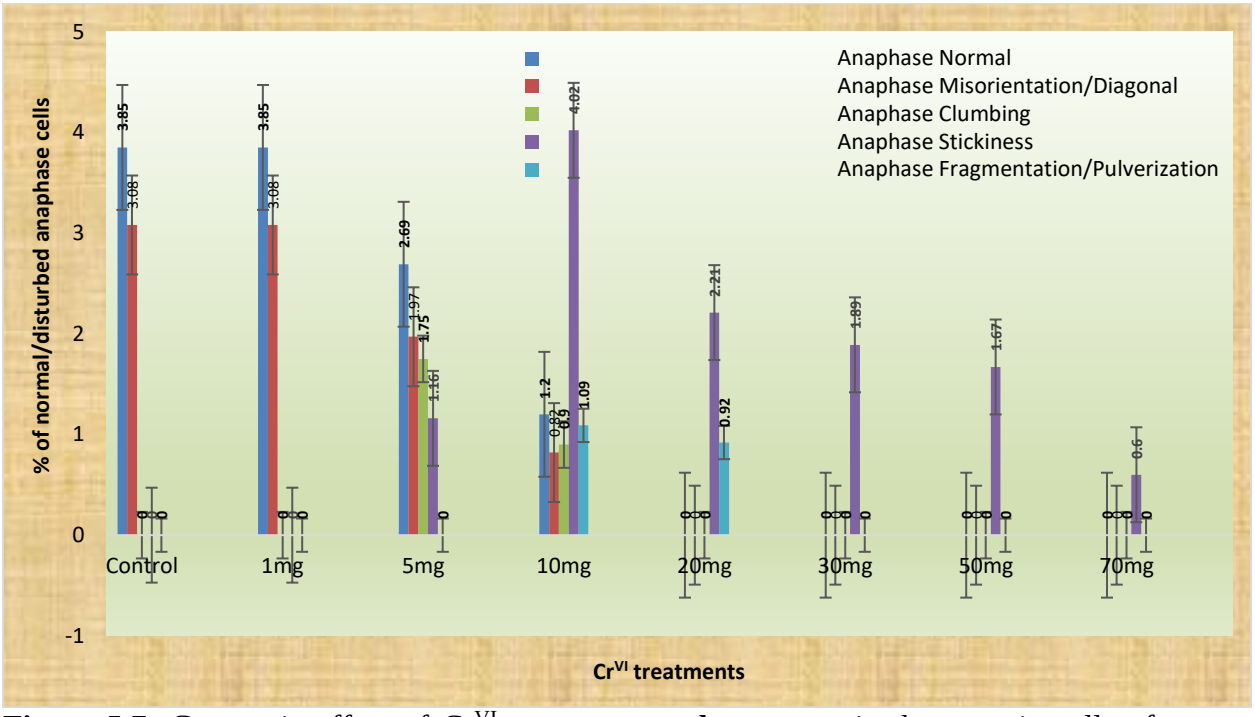

Figure 5.7: Genotoxic effect of Cr^{VI} stress at **anaphase stage** in the root tip cells of *Amaranthus dubius*

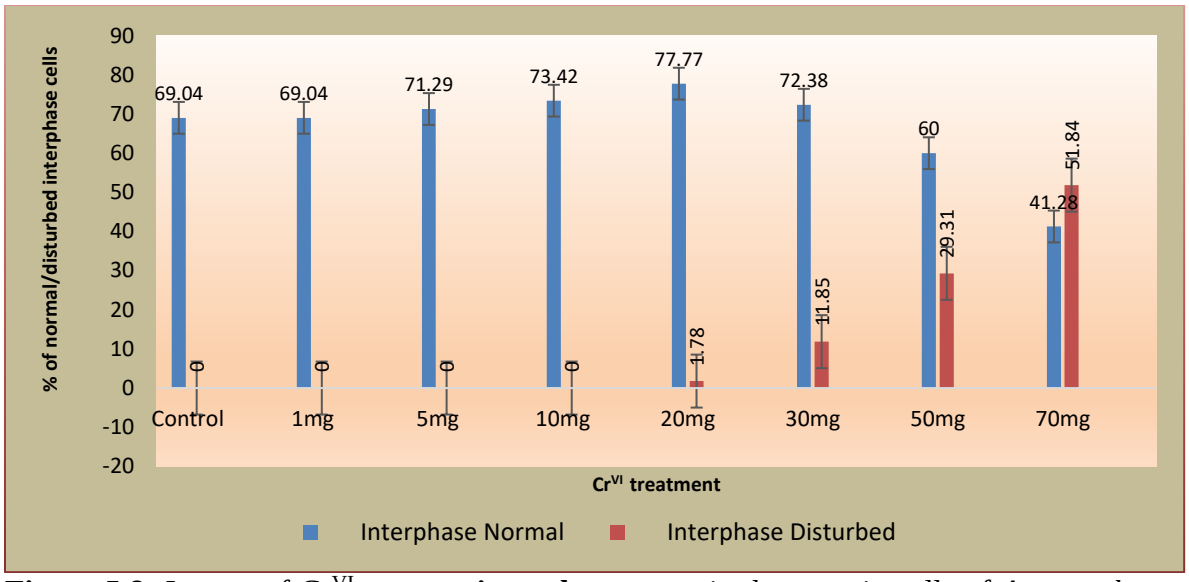

Figure 5.8: Impact of Cr^{VI} stress at **interphase stage** in the root tip cells of *Amaranthus dubius*

The data recorded in tables 1- 8 regarding the percent of total abnormality induced by the toxicity of different concentrations of Cr^{VI} in the root tip cells of *A. dubius* shows all treatments have contributed to an increase in total abnormality percent compared to control treatment. The total abnormality percent constituted by chromosomal and non-chromosomal abnormalities exhibited direct relationship with increase in concentration of Cr^{VI}. The increase is more prominent at higher concentrations with maximum value recorded in 70mg concentration of Cr^{VI} (56.21%) and is 55.9% increase over control value (0.31%). The direct relationship of abnormality percent with increase in concentration of the Cr^{VI} in *A. dubius* clearly indicates dose dependent genotoxicity or cytotoxicity (Figure 5.9). This could be due to the increase in mitotic poisoning with increase in concentration of Cr^{VI} which interferes more with nucleic acid metabolism and the protein which form an integral part of the chromosomes (EI-Sadek, 1972). This could also be due to the blocking of normal onset of prophase together with increased nuclear disturbances at interphase cells. The statistical analysis of total abnormality percent obtained in *A. dubius* revealed that, with the exception of initial level Cr^{VI} stress of 1mg and 5mg treatments, the increase in the abnormality percent induced by all the other stress treatments is significantly higher over the control (Table 5.9). The highest abnormality percent induced by 70mg Cr^{VI} stress differed significantly higher over all the other Cr^{VI} stress while with respect to 50mg Cr^{VI} stress, it is

significantly lower compared to 70mg Cr^{VI} stress whereas it differed significantly higher over the rest.

Table 5.9: Duncan analysis of total abnormality percent obtained in *Amaranthus dubius* under Cr^{VI} stress

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05					
	1	2	3	4	5	6
1 (Control)	0.31					
2 (1mg)	4.98	4.98				
3 (5mg)	9.99	9.99	9.99			
4 (10mg)		14.03	14.03	14.03		
5 (20mg)			15.48	15.48		
6 (30mg)				23.57		
7 (50mg)					37.07	
8 (70mg)						56.21
Sig.	.060	.078	.282	.064	1.000	1.000

Means for groups in homogeneous subsets are displayed.

The values of mitotic index are highest in the root tip cells treated in 1mg/litre concentration of hexavalent chromium (30.96%) while the lowest value (6.88%) has been recorded in 70mg/litre concentration. Mitotic index value for control is 29.34% which is slightly less than (5.23%) the root tips treated with 1mg/litre concentration whereas all the remaining treatments recorded values lower than the control. The mitotic index value at the highest concentration of 70mg Cr^{VI}/litre is found reduced by 76.89% compared to control. The study observed that the number of dividing cells decrease with increase in concentration of hexavalent chromium treatment. Therefore, the investigation reveals a dose dependent reduction of mitotic index in the treated root tip cells of *A. dubius* and the reductions are more predominant at higher concentrations (figure 5.9). The reduction in number of dividing cells and the decrease in mitotic index as the concentration of hexavalent chromium (Cr^{VI}) increased indicate mitodepressive activity of Cr^{VI} and this can be attributed to the capacity of Cr^{VI} in blocking the G1 stage which affects the suppression of DNA synthesis and nuclear proteins (Mohandas & Grant, 1972; Yekeen *et al*., 2011). The Duncan analysis on the mitotic index of *A. dubius* obtained under different levels of Cr^{VI} stress revealed the differences in the mitotic index values induced at comparatively lower levels of 1mg, 5mg and 10mg concentrations are insignificant

over the value of control. The highest reduction in mitotic index value recorded by 70mg Cr^{VI} showed no significant differences over the immediate stress treatment of 50mg Cr^{VI}; however, the reduction induced by both the Cr^{VI} stress are significantly differed over the rest of the stress treatments (table 5.10).

Table 5.10: Duncan analysis of mitotic index obtained in *Amaranthus dubius* under Cr^{VI} stress

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	6.88				
7 (50mg)	10.70	10.70			
6 (30mg)		15.76	15.76		
5 (20mg)			20.45	20.45	
4 (10mg)				26.58	26.58
3 (5mg)				28.71	28.71
1 (Control)					29.36
2 (1mg)					30.96
Sig.	.333	.200	.235	.051	.312

Means for groups in homogeneous subsets are displayed.

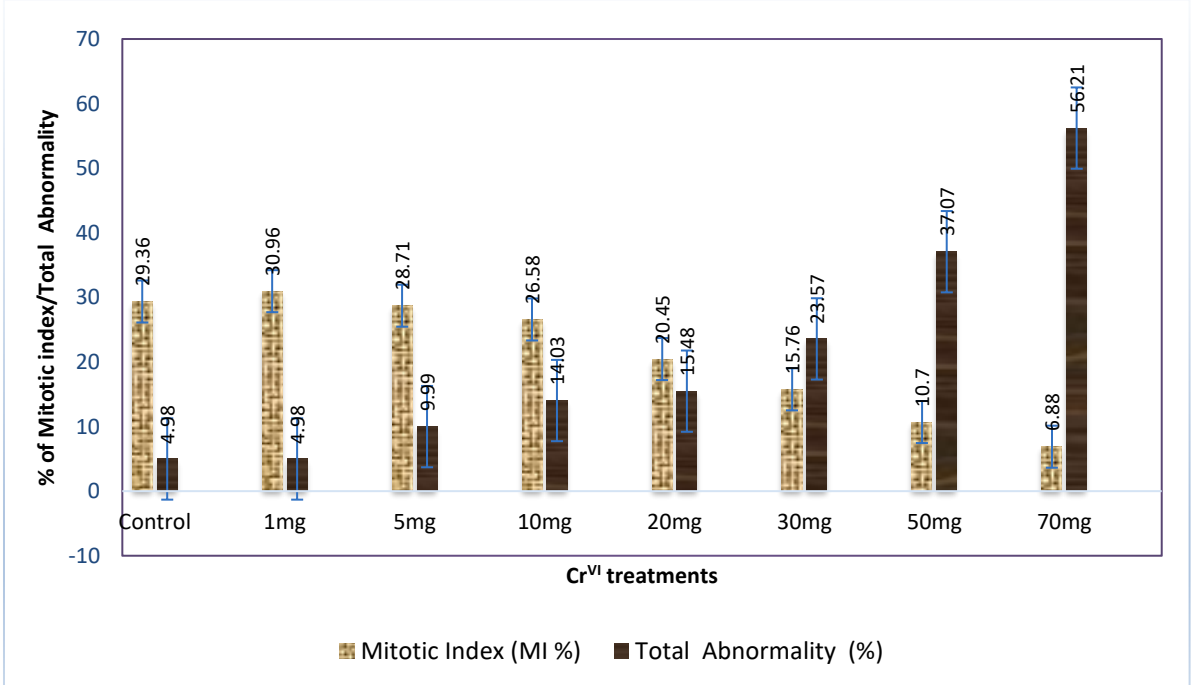

Figure 5.9: Impact of Cr^{VI} stress on Mitotic index and Total Abnormality percent in the root tip cells of *Amaranthus dubius*

CONCLUSION

The various chromosomal aberrations and the cytological abnormalities observed and recorded in the study clearly revealed the genotoxic and cytotoxic potentiality of hexavalent chromium (Cr^{VI}) in the root tip cell of *Amaranthus dubius.* The study further revealed that the hexavalent chromium stress has dose dependent genotoxic and cytotoxic effect. With the increasing concentration of Cr^{VI} stress, the percent of dividing cells and mitotic index in the root tip cells of *A. dubius* generally tend to reduce while the overall percent of abnormality tend to increase. This inference in the study suggests the intensity of Cr^{VI} stress is negatively correlated with the mitotic index whereas it is positively correlated with the induction of abnormality percent. The genotoxic and cytotoxic effects of Cr^{VI} stress obtained in the study when related with the inferences of previous chapters, it clearly indicates some correlations. The hexavalent chromium stress induced production of reactive oxygen species (ROS) and its activity disrupted the genome stability and chromosomal behavior in *A. dubius* which may have caused impaired gene expression and protein production. This might be the reason for various physiological and biochemical disorders inferred in previous chapter which in turn might have contributed to the reduction in morphological growth and development. The types and the frequency of chromosomal aberrations and other cytological abnormalities and the degree of mitotic inhibitions inferred in *A. dubius* in response to different levels of Cr^{VI} stress may be useful as indicators in future with respect to the assessment of metal Cr^{VI} pollution status in the area. The present study results further demonstrate that the cytological analysis using *A. dubius* is a useful screening test like Allium test for the evaluation of toxicity of heavy metal. Leafy vegetables are generally known to accumulate higher amounts of heavy metals in their leaves than in roots and stems and as the metal pollutants are non-degradable and are readily taken up by crop plants, these are likely to enter easily into the food chain causing serious health hazards to human beings more or less comparable to plants. The intension of the study is not to scare the consumer but to create awareness on the need to be inquisitive on the sources of the vegetable being brought for consumption.

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CHAPTER-6

IMPACT OF HEXAVALENT CHROMIUM (Cr^{VI}) STRESS ON MACRO AND MICRONUTRIENT ELEMENTAL UPTAKE AND BALANCE

INTRODUCTION

It is desirable to have metals or trace elements to ensure the different metabolic and physiological pathways in the course of development and growth of plants. Nevertheless, a variety of essential and non-essential metals are available. Those metals which are essential in trace for plant growth include zinc (Zn), iron (Fe), Copper (Cu), manganese (Mn), nickel (Ni) etc. but when available in excess concentration, it may turn out to be extremely toxic (Chaffai & Koyama, 2011). However, it is identified that non-essential metals such as chromium (Cr), cadmium (Cd), lead (Pb), aluminum (Al), mercury (Hg), arsenic (As) have no biological activity in plants and even in very low quantities may become quickly and critically toxic (Boyd & Rajakaruna, 2013). The heavy metal chromium (Cr) is considered to be a hazardous ion, which has a negative impact on plant growth by weakening the vital metabolic processes, interrupting the mineral nutrition and many other physiological processes. It is observed that due to the impact of heavy metal stress in the growth environment, there occurs a disturbance in the uptake and balance of essential elements by hiding the sorption sites and resulting in formation of insoluble complexes (Kabata & Szteke, 2015; Osu & Onyema, 2016). Moreover, the mineral nutrition in plants is affected in a complex manner due to the similarity in structure among chromium and some of the essential elements. It is identified that the metal chromium, when available at higher concentration, displaces certain essential elements from their physiological binding sites by competing with them and eventually cause reduction in the uptake and translocation of those key elements (Oliveira, 2012). The uptake of essential elements like Mn, Cu, Zn, Fe, Ca and Mg is observed to be disrupted due to the impact of chromium metal (Turner & Rust, 1971; Moral *et al*., 1995; Chatterjee & Chatterjee, 2000; Gardea *et al*., 2004; Zeng *et al*., 2010). When exposed to excess chromium stress, there is a gradual reduction in the intake of micronutrients like Zn, Cu, Fe, Mn and macronutrients like potassium (K), phosphorous (P) and nitrogen (N)

from soil as noted by Sundaramoorthy *et al*. (2010) in *Oryza sativa*. At the same time, there are some conflicting reports also on the impact of Cr stress on the uptake of several elements. The enhanced chromium accumulation in different regions of *Citrullus vulgaris* is identified to improve the uptake of manganese (Mn) and phosphrous (P) causing their content increase in the leaves (Dube *et al*., 2003). Similar observation has also been reported in *Lolium perenne* by Vernay *et al*. (2007). A higher positive correlation between Fe and Cr concentration in Veronica plant species has been inferred by Zivkovi´c *et al*. (2012) and a similar correlation between Cr^{VI} and Mg concentration in *Oryza sativa* has been reported by Zeng *et al*. (2010). It is generally suggested that Cr stress interferes with the functions of mineral nutrients in plants, thus causing serious inhibition of plant growth (Adriano, 1986). The interaction of heavy metal chromium with the uptake and accumulation of other inorganic nutrients have received significant attention by the investigators and researchers. In the light of this information, the objective of this chapter is to analyse the impact of different concentrations of hexavalent chromium (Cr^{VI}) stress on the uptake and balance of various nutrient elements in *Amaranthus dubius*. The study further aims to reveal whether any correlation exist between the change in nutrient uptake and balance impacted by Cr^{VI} stress and the inference obtained with respect to the growth parameters of *A. dubius*.

REVIEW OF LITERATURE

The macro and micro nutrients vital for the plant growth, if available in the growth environment, contribute to the proper development of plants. In other words, nutrient deficiency can retard plant growth. Out of the many factors that influence the uptake of minerals by plants via roots from the growing environment, one factor is the presence of heavy metals in excess quantity that are insignificant in plant metabolic and physiological processes (Cataldo & Wildung, 1978). During the absorption of the essential minerals by plant roots, the heavy metals around the roots also get accumulated into plants. In accordance with the Hard–Soft Acid– Base (HSAB) theory proposed by Pearson, it is understood that most of the non-essential heavy metals exhibit identical properties same as the necessary minerals like Mg^{2+}, Fe^{2+} etc. (Housecroft & Sharpe, 2005). The presence of Cr^{3+} in the growth environment can result in a competition with some of the key nutrients for the uptake by the roots, causing a disruption in the absorption of these nutrients. Gill (2014) has observed that the heavy metal toxicity in plants is dependent on the variety of plant and heavy metal. Therefore, the impact of Cr accumulation on the absorption of essential nutrients by plants is to be analysed in detail.

It is clearly identified that metal chromium (Cr) is an environmental pollutant with high toxicity which can retard the growth and development of plant community (Panda & Choudhury, 2005; Monalisan & Kumar, 2013). The two variants of chromium identified in plants are trivalent Cr^{3+} and hexavalent Cr^{6+}, of which the latter is highly toxic compared to former. Cr^{6+} gets converted to Cr^{3+} due to reduction which can cause a change in soil pH to extreme alkalinity or acidity based on the existing soil subsurface conditions and upset the bioavailability and sorption of nutrients by plants (Hawley *et al*., 2004). The reasons for the decrease in the uptake of nutrients may be the reduced translocation of essential elements caused by the dislocation of nutrients from physiologically crucial binding sites or declined root growth and impaired root penetration under Cr stress (Shahzad *et al*., 2018; Mengel & Kirkby, 1987). The retarded root growth and impairment in the root penetration into the soil as a result of Cr toxicity may be the reason for reduction in the N, K, P and similar elements in plants (Arun *et al*., 2005).

Due to the similarity in the physical and chemical properties of heavy metal Cr with key essential elements like Zn, Cu, Mn etc., there occurs a competition among them to occupy the binding sites of metal absorptive and enzymatic proteins, particularly when the Cr uptake by plants is more due to its high concentration (Bridges & Zalups, 2005). This may lead to a deficiency in the vital nutrients thereby affecting the functions of Mn/Zinc/Copper-dependent enzymes like Mn- dependent superoxide dismutase (MnSOD) (Millaleo *et al*., 2010), copper/zinc- dependent superoxide dismutase (Cu/Zn SOD) etc. resulting in oxidative damage owing to heavy metal toxicity (Zhai *et al*., 2014). Heavy metal chromium, higher in concentration, can cause displacement of essential nutrients from the crucial physiological binding sites resulting in reduced uptake of these necessary elements which indicates the fact that Cr^{VI} toxicity may be the reason for deficiency of essential nutrients in plants (Moral *et al*., 1996).

Iron (Fe), among all micronutrients, is the most critically affected one in its uptake due to heavy metal stress as a result of its similarity in chemical structure, behaviour and presence in soil with other heavy metals and it is identified that excess concentration of heavy metals induces chlorosis in tender leaves mainly due to deficiency of iron (Schaaf *et al*., 2006; Morrissey *et al*., 2009; Wu *et al*., 2012). Iron deficiency symptoms and its induced responses are often mistaken during heavy metal treatments in plants (Meda *et al*., 2007; Fukao *et al*., 2011). The primary root length is more forbidden due to iron deficiency than higher concentration of cadmium which may be attributed to the concomitant reduction in Ca level much lower than the crucial deficiency levels causing strong suppression of primary root elongation (Gruber *et al*., 2013). Iron is a constituent of many essential enzymes like cytochromes found in electron transport chain and is indispensable in most of the biological functions including photosynthesis. Fe is needed for synthesis of chlorophyll and for the proper functioning and maintenance of chloroplast structure in plants.

Manganese (Mn) is identified as one of the micronutrients essential for plants to carry out various metabolic and growth functions such as respiration, photosynthesis and biosynthesis of enzymes such as isocitrate dehydrogenase, malic enzyme, nitrate reductase and isocitrate dehydrogenase (Todorovi´c *et al*., 2009). It is a cofactor needed

for a several plant enzymes like Mn- dependent superoxide dismutase (Millaleo *et al*., 2010). In addition, manganese plays a pivotal role in the metabolism of nitrogen and carbohydrate, synthesis of acyl lipids, fatty acids and carotenoid together with hormonal activation (Lopez-Mill´an *et al*., 2005). Manganese also contributes to the proper functioning of photosystem II (PSII) particularly in the course of breaking of water molecules into oxygen (Arya & Roy, 2011) and also protects PSII from photo damage (Hou & Hou, 2013).

Copper (Cu) is an important and vital micronutrient that takes part in several physiological activities of plants such as acting the role of catalyzer during the redox reaction in chloroplasts, mito- chondria, and cytoplasm of cells (Fargasova, 2004) and works as an electron carrier at times of plant respiration (Yruela, 2009). Zinc (Zn) is another key trace metal which is a basic constituent of distinct proteins named as zinc fingers that bind to DNA and RNA and participate in their regulation and stabilization (Gupta *et al*., 2012). Further, Zn forms a major constituent of many enzymes like oxidoreductases, transferases, and hydrolases (Mishra & Dubey, 2005) and ribosome, in addition to contributing to root growth and formation of carbohydrates and chlorophyll (Kleckerova, 2011).

Magnesium (Mg^{2+}), being the central core of chlorophyll molecule in all plant tissues, its deficiency can cause insufficiency of chlorophyll leading to stunted growth in plants. The various metabolic activities and reactions that are influenced by magnesium include CO_2 fixation in photosynthesis, chlorophyll biosynthesis, photophosphorylation, protein synthesis, phloem loading, partition and utilization photo assimilation, photooxidation on leaf tissue and formation of reactive oxygen species (Cakmak & Yazici, 201). Based on Hard–Soft Acid– Base (HSAB) theory by Pearson, Cr^{3+} and Mg^{2+} have the same properties (Housecroft & Sharpe, 2005). Under this circumstance, the availability of Cr^{3+} in the growth environment results in an enhanced competition for absorption of Mg^{2+} by roots thereby causing a disruption. Magnesium deficiency can escalate the antioxidant enzyme functions and reduce the chlorophyll content of leaves as reported by Chou *et al*. (2011). The competition between chromium and magnesium in their absorption may result in the reduction in the chlorophyll content in vegetative plants for the reason that

magnesium is essentially required for biosynthesis of chlorophyll, in addition to nitrogen and iron (Sulistiani *et al*., 2021). In plants that are cultivated in media contaminated with heavy metal chromium, the absorption of magnesium and content of chlorophyll is dependent on the plant species, availability of other heavy metals and the nature of the growth media (Sulistiani *et al*., 2021).

Calcium (Ca^{2+}) is identified as a vital macronutrient for plant growth and carries out many essential functions including regulation of cell turgidity, performs selective permeability in cell membrane, acts as counter-cation for inorganic and organic anions in the vacuole which is needed for structural roles in both cell wall and cell membrane etc. The co-existence of Ca^{2+} ions in the soil have a commendable impact on the plant bioavailability of heavy metals. The diameter and valency of calcium cation available in the soil is similar to that of many heavy metals like Cr, Pb, Cd etc. which may cause a reduction in the uptake of the calcium from the soil via ion exchangeability (Eller & Brix, 2016). Recent studies have shown that excess concentration of Ca in soil may cause an extreme alleviation and neutralization of heavy metal stress in most of the Fabaceae species (Avelar *et al*., 2013). Plants subjected to heavy metal stress are noted to display higher intracellular Ca^{2+} levels thereby inducing better adaptive measures so as to mitigate the toxicity of heavy metals (Yang & Poovaiah, 2003). A study conducted in *Brassica juncea*, in line with the above observation, proved that the application of Ca^{2+} lessened the toxic effect and enabled the plant to withstand the harmful effects of heavy metal (Ahmad *et al*., 2015). Furthermore, experiments conducted on various plant species illustrate that external application of calcium and silica, Ca^{2+} and/or K^{2+} and calcium and spermidine can aid in the easing of heavy metal toxicity and bring down the accumulation of metals (Siddqui *et al*., 2012; Gong *et al*., 2016). Tolerance to heavy metal stress mediated via Chromium (Cr) was noticed to be improved in foxtail millet (*Setaria italica*) due to the interaction of hydrogen sulfide with Ca^{2+}. It is observed that Ca^{2+} is able to provide better tolerance to Cr stress by increasing the antioxidant enzyme activity (Fang *et al*., 2014).

Nitrogen (N_2) is identified as the most vital component contributing to plant growth and development. It forms a major constituent of nucleic acids, chlorophyll, protoplasm and

amino acids in plants. It is identified by Sangwan *et al*. (2014) that, in Cluster bean (*Cyamopsis tetragonoloba* L.), there is a gradual and prominent reduction in nitrogenase function at various growth stages with increasing concentration of Cr. A reduction in nitrogen fixation has been observed in *Pisum sativum* in response to presence of heavy metal Cr (Bishnoi *et al*., 1993). The root and shoot component of Cluster bean showed reduced nitrogen content with increased concentrations (2.5, 5, 10, 25, 50, 75 and 100 mg/l) of chromium (Sangwan *et al*., 2014). Retardation in nitrogen metabolism and nitrogen content has also been identified in cowpea plant due to the availability of metals in excess concentrations (Mayz & Cartwright, 1984). The uptake and assimilation of nitrogen is adversely affected in wheat leaves causing a reduction in its concentration, as a result of Chromium treatments (Sharma & Mehrotra, 1993). Nitrogen intake from soil is inhibited in sorghum owing to the availability of heavy metals (Gomes *et al*., 1985). Heavy metal Cr has had an adverse impact on the uptake of nitrogen (N), potassium (K) and Iron (Fe) as well as stimulated the uptake of Mn and Ca and decreased the translocation of elements like P, Cu, Zn and Fe from roots to shoots in bush bean as reported by Barceló *et al*. (1985). The uptake of NH^{4+} by roots has been considerably restricted under heavy metal stress in many plants, mostly caused by perturbations in active uptake process of ATPase (Weber *et al*., 1991). In Silene vulgaris, a comparatively reduced or suppressed NH^{4+} uptake is noticed due to the presence of Cu (Weber *et al*., 1991). In cucumber seedlings also, a demised intake of NH^{4+} is observed due to the toxicity caused by heavy metals like Cd^{2+}, Pb^{2+}, and Fe^{2+} (Burzynski & Buczek, 1998). Many other stress imparting metals such as Pb, Cd, Cr, Ni, Zn, and Mn are also identified to affect the uptake of NH^{4+} in various plant species.

Potassium (K^+) is an inevitable macronutrient for growth and development of plant species. It activates many key enzymes which are involved in protein synthesis, photosynthesis, N and C metabolism and sugar transport. K also plays a vital role in the opening and closing of stomata as well as transportation of water and nutrients. The enhanced presence of chromium in the growth media has identified to result in reduced potassium content in the shoot and root of paddy (Sundaramoorthy *et al*., 2010). A similar observation is noticed in *Trifolium repens* (Lee & Pritchard, 1984), in *Pisum sativum* (Matsumoto & Yamaya, 1986), in corn (Gerzabek & Edelbauer, 1986) and

in *Oryza sativa* (Lidon, 1993). The exercise of a regulatory command on the uptake of potassium may be the result of the competition between chromium and potassium ions during the absorption process of nutrients by the plant roots (Chaney, 1978).

Yet another significant macronutrient indispensable for plant growth is Phosphorus (P). It plays a major role in energy storage and transfer, photosynthesis, cell division, the metabolism of sugars and cell enlargement. It is an essential component of nuclear protein and ATP. A retardation in the phosphorous content is observed in paddy root and shoot with increasing concentrations of Cr (Sundaramoorthy *et al*., 2010). The uptake of phosphorous is noticed to decrease due to heavy metal stress in soybean (Walker *et al*., 1979). Study conducted in *Phaseolus aureus* cultivars by Varshney (1990) and in *Convolvulus arvensisn by* Gardea *et al*. (2004) identify a reduction in the accumulation of phosphorous nutrient due to toxicity induced by heavy metals. The depletion in the intake of phosphorous in paddy plant when subjected to chromium stress may be the result of competition between the two elements either during the process of uptake or transport of phosphorus by the root system (Swamy, 1998). The toxicity of chromium in soil may affect the root cells resulting in an impairment in the absorption of phosphorous and also cause a reduction in the availability of phosphorous in soil (Dahiya *et al*., 2003).

Cr stress has a large impact on the plant biomass and nutrient uptake. Cr interferes in a complex way with the intake of vital nutrients. The heavy metal Cr is identified to be in competition with Fe for the binding sites (Chatterjee & Chatterjee, 2000) thereby interfering with Fe absorption causing a decrease in its accumulation which adversely affects the biosynthesis of chlorophyll and heme (Gopal *et al*., 2009). Fe metabolism is impaired as a result of Cr toxicity due to the interference of Cr with the Fe availability leading to its reduced concentration in the plant tissues (Gopal *et al*., 2009). Turner and Rust (1971) opined that as a result of exposure to chromium, the biomass of *Glycine max* is reduced which may be attributed to the interference of chromium in P metabolism and confined incorporation of S in some crucial amino acids. Heavy metal chromium is noted to compete with nutrient P for surface sites and with other elements such as Fe, S and Mn for transport binding sites and thus effectively competing with almost all nutrients for gaining entry into the plant body (Arun *et al*., 2005). Moral *et al*. (1996) conducted an

elaborate study on the content of mineral nutrition in tomatoes which are subjected to Cr toxicity and observed a negative effect on absorbed Fe content with increasing concentration of Cr. The concentration of Mn and Cu has been noticed to decrease with increase in Cr level and Zn concentration is identified to decrease in leaves and flowers but increased in root and stem (Sharma & Pant, 1994). Altogether, Moral *et al*. (1996) opined that the uptake of many essential nutrient elements has been reduced due to the competitive binding by Cr^{VI} to common carriers.

Sujatha *et al*. (1996) reported that irrigation using tannery effluents with excessive levels of Cr caused deficiency of micronutrients in many agricultural plants. Samantaray *et al*. (1998) observed that heavy metal chromium interferes with the absorption as well as accumulation of a number of metals and nutrients like Fe, Mn, Ca, Mg, K, and P, both in the shoot and root system of plants, substantially causing decreased tissue and cellular concentration. Inhibition in the functions of plasma membrane H^+ ATPase may be one of the reasons for reduced intake of nutrients by plants subjected to chromium stress (Shanker *et al*., 2005). Chromium may interact with other essential elements because of its varying valences thereby exhibiting its toxicity (Turner & Rust, 1971). Reduction in plant biomass is another significant impact caused by chromium induced variations in the ion concentration in plants. Both the biomass content and intake of nutrients are largely affected in plants when under Cr stress (Harminder *et al*., 2013).

In general, plants under Cr stress have shown decreased absorption of both micro elements and macro nutrients as reported by Gardea *et al*. (2005). The uptake of K^+ and H^+ is obstructed by chromium in *Zea mays* roots, indicating an interference with the transport functions of plasma membrane (Zaccheo *et al*., 1985). The mineral constituents, mainly Fe, Ca, and Mg are affected negatively in *Lolium perenne* when exposed to Cr^{VI} as observed by Vernay *et al*. (2007). *Oryza sativa*, which is grown in fields irrigated with various concentrations of Cr (50–500 mg/kg), indicated a steady decrease in the uptake of macronutrients (K, P, and N)) and micronutrients manganese (Mn), zinc (Zn), copper (Cu) with increasing levels of Cr (Ahmad *et al*., 2011). Spinnach showed a decreasing trend in Fe concentration under Cr stress (Gopal *et al*., 2009) and same observation has been recorded in sunflower also (Gupta *et al*., 2000). Fe concentration has been adversely

affected in *Brassica oleracea* at excessive levels of Cr and causing translocation of Zn, Cu, S, P, Mn from the roots to other parts of the plant (Chatterjee & Chatterjee, 2000). Cr toxicity has resulted in diminished uptake of elements like K, P, Mg, and Cu via roots and Ca, Fe, and Cu in the leaves of S. kali (Harminder *et al*., 2013). When exposed to Cr, Liu *et al*. (2008) observed a decreased uptake of micronutrients Mn, Fe, Cu, and Zn in *Amaranthus viridis*. It is demonstrated that chromium toxicity may cause a reduction in the uptake of many essential nutrient elements like Fe, K, P, Mg, Mn etc. (Skeffington *et al*., 1976). In Soybean crop grown in soil with chromium toxicity, a decrease in Ca, K, Mg, P, Cu and B concentrations has been noticed but uptake of Fe, Mn and Zn was not identified to be affected (Turner & Rust, 1971).

Chromium is observed to disturb the nutrient balance in Citrullus plants whereby the transporting of chromium to its different parts has caused an enhancement in the concentrations of Mn and P and reduction in S, Cu, Zn and Fe in the leaves (Dube *et al*., 2003). Turner and Rust (1971) also suggested an identical effect in the intake of several nutrients in plants subjected to Cr toxicity. An increase in the levels of P and Mn and decrease in the levels of Fe, Cu, Zn, and S contents in leaves of Watermelon plants has been observed when grown in medium with excess of chromium (Dube *et al*., 2003). Fe availability and absorption by plants is improved when the soil is supplied with Cr^{VI} or Cr^{III} or tannery waste (Cary *et al*., 1977; Barcelo et al., 1993). Nutrients like P and Zn are identified to have an enhanced accumulation in *Citrullus vulgaris* when exposed to Cr^{VI} (Dube *et al*., 2003).

MATERIALS AND METHODS

The young shoot component of *Amaranthus dubius* plants treated with different concentrations of hexavalent chromium (Cr^{VI}) and control were analysed on the 45th day after the treatment. The various parameters considered for the analysis include macronutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) & magnesium (Mg) and micronutrients such as iron (Fe), zinc (Zn), copper (Cu) & manganese (Mn) and the heavy metal chromium (Cr).

Estimation of available nitrogen (NH_4^+, NO_3^-) Alkaline permanganate method (Subbiah & Asija, 1956)

Principle: The amount of soil nitrogen released by alkaline permanganate solution was estimated by distillation with sodium hydroxide. The distillate was collected in boric acid containing mixed indicator and titrated against standard acid – H_2SO_4. The nitrogen so estimated was designated as available nitrogen which was correlated with crop response to nitrogen fertilization.

Reagents:

Liquid paraffin or paraffin wax, potassium permanganate solution, NaOH solution, distilled water, boric acid with mixed indicator, N / 50 sulphuric acids

Procedure:

20g of the soil was weighed and transfered to a distillation flask. 20 mL of distilled water and 1 mL of liquid paraffin or 1 g of paraffin wax was added to control frothing. A few glass beads were put to prevent bumping and then 100 mL of 0.32% potassium permanganate solution and 100 mL of 2.5% NaOH solution were added. The contents were distilled in a steady rate and the liberated ammonia was collected in a 100 mL conical flask containing 20 mL of 2% boric acid with mixed indicator (0.5 g of bromocresol green, 0.1 g of methyl red in 100 mL of ethyl alcohol). The distillation was continued for about half an hour or until 100 mL of distillate was collected in the beaker. The ammonia collected was titrated against N / 50 sulphuric acids.

Calculation:

1 ml of N/50 H_2SO_4 = 0.0028 g of Nitrogen

$$N\ Kg\ ha^{-1} = \frac{TV \times 0.0028 \times 1000 \times 2.24 \times 10^6}{1000 \times 20}$$

Estimation of available phosphorus (**H_2PO_4**) - (Olsen & Watanabe, 1954).

Reagents:

Ammonium fluoride (NH_4F) 1N: 37 g of NH_4F was dissolved in distilled water and the solution was diluted to 1 litre. The solution was stored in polythene bottle.

Hydrochloric acid (HCl) 0.5 N: 20.2 mL of concentrated HCl was diluted to a volume of 500 mL with distilled water.

Bray No.1: 15 mL of 1N ammonium fluoride (NH_4F) and 25 mL of 0.5N Hydrochloric acid (HCl) was added to 460 mL of distilled water. This solution can be kept in glass for more than one year.

Ammonium paramolybdate ($(NH_4)6Mo_7O_{24}.4H_2O$): 12g of ammonium paramolybdate was dissolved in 250 mL of distilled water. 0.2908 g of potassium antimony tartartate ($KSbO.C_4H_4O_6$) was dissolved in 100 mL of distilled water. These dissolved reagents were added to 1 litre of 5N sulphuric acid (H_2SO_4) (141 mL of concentrated H_2SO_4 diluted to 1 litre), mixed thoroughly and diluted with distilled water to 2 litres. It was stored in a Pyrex glass bottle in a dark and cool compartment (Reagent A).

Ascorbic acid: 1.056 g of ascorbic acid was dissolved in 200 mL of reagent A and mixed. This ascorbic acid (reagent B) should be prepared as and when required because it cannot be kept for more than 24 hours.

Standard phosphate solution: 0.4393 g of oven-dry AR grade potassium dihydrogen phosphate (KH_2PO_4) was dissolved in distilled water and diluted the solution to 1 L. 1ml of this solution contains 100 µg of P. From this solution, a secondary standard of 2 µ g mL^{-1} was prepared by pipetting out 2 mL and diluted to 100 mL with distilled water.

Procedure:

Extraction: Available P in acidic soils was extracted using Bray No.1 (Bray & Kurtz, 1945) which consists of 0.03 N NH_4F and 0.025 N HCl. The combination of HCl and NH_4F was designed to remove easily acid soluble P forms, largely calcium phosphates and a portion of the aluminium and iron phosphates. The NH_4F dissolved aluminium and iron phosphates by its complex ion formation with these metal ions' acid solution.

5 gm of soil was weighed out into a 100 mL conical flask and 50 mL of Bray No.1 reagent was added and shaken for exactly 5 minutes. It was filtered through Whatman No.42 filter paper. To avoid interference of fluoride, 7.5 mL of 0.8 M boric acid (50 g of H_3BO_3 per litre) can be added to 5 mL of the extract if necessary. The phosphorus in the extract was estimated by ascorbic acid method (Olsen & Watanabe, 1954).

Estimation by reduced molybdate blue colour method:

5 mL of the extract was pipetted into a 25 mL volumetric flask and diluted to approximately 20 ml. 4 mL of reagent B was added. The volume was made up with distilled water and the contents shaken well. The intensity of colour was read after 10 minutes at 660 nm. The colour was stable for 24 hours and the maximum intensity developed within 10 minutes. The concentration of P in the sample was computed from the standard curve.

Preparation of standard curve:

Different concentrations of P was prepared by taking 1, 2, 3, 4, 5 and 10 mL of G mL^{-1} P solution in 25 mL volumetric flasks. 5 mL of extracting reagent (Bray No.1) was added and colour developed as described above by adding reagent B. The concentration vs. absorbance curve was plotted on a graph paper.

Calculation:

$$\text{Available P (mg kg}^{-1}\text{ soil)} = \mu\text{g PmL}^{-1}\text{ of aliquot} \times 50/5 \times 25/5$$

$$\text{Available P (mg kg}^{-1}\text{ soil)} = \frac{\text{Absorbance for sample} \times 50}{\text{Slope of Std. curve}}$$

$$\text{Available P (kg ha}^{-1}\text{ soil)} = \text{Available P (mg kg}^{-1}\text{ soil)} \times 2.24$$

Estimation of available potassium (K^+) - (Jackson, 1973).

Principle:

By definition, exchangeable K is that which is free to exchange with cations of salt solutions added to soils, but the quantity exchanged from the soil depends on the nature of replacing solution. Hence with reference to fertility evaluation exchangeable K is defined more specifically as that which is extracted with neutral one N ammonium acetate minus the water-soluble K. In normal soil, as the water-soluble K is so small there is no appreciable error even if it is included in the water soluble plus exchangeable K which represents the available pool. The removal of water-soluble K before extraction with ammonium acetate is not recommended because as the salt content of soil solution is decreased, the adsorption of divalent cations in solution increases.

Reagents:

Neutral 1 N ammonium acetate solution (CH_3COONH_4): 77.08 gm of ammonium acetate was dissolved in distilled water and made up to 1 L. The pH was adjusted to 7 with acetic acid or ammonium hydroxides.

Standard K solution: 1.908 gm of dried K chloride (KCl) AR grade was dissolved in distilled water and made up to 1 L so as to get 1000 µg mL^{-1} K solution.

Procedure:

Extraction: Available potassium was extracted by shaking with neutral normal ammonium acetate for 5 minutes (Hanway & Heidel, 1952). 5gm of soil was shaken with 25 mL neutral normal ammonium acetate for five minutes and immediately filtered through a dry Whatman No: 42 filter paper. First few mL of the filtrate was discarded. K concentration in the extract was determined using flame photometer after necessary setting and calibration of the instrument (Jackson, 1973).

Standard curve for potassium:

Measured aliquots was diluted from the standard solution using ammonium acetate solution to give concentrations of 5 to 20g K. After attaching the appropriate filter and adjusting the gas and air pressure, the reading was set in the flame photometer to zero for the blank (Ammonium acetate) and at 100 for 20g K. The curve was obtained by plotting

the reading against different concentrations (4, 10, 15, 20g) of K. Fluctuation in gas and air pressure does not allow steady reading in the meter and must be taken care of.

Calculation:

Available K (mg kg^{-1} soil) =µg KmL^{-1} of the aliquot x 25/5

Available K (mg kg^{-1} soil) =µg KmL^{1} of the aliquot × 5

Available K (kg ha^{-1}) = Available K (mg kg^{-1} soil) x 2.24

Estimation of available calcium (K^+) and magnesium (Mg^{2+})- (By Atomic Absorption Spectrophotometry)

Principle:
In the absence of excess of $CaCO_3$, the cations Ca^{2+}, Mg^{2+} and Na^+ along with K^+ appear to be completely exchangeable by neutral normal ammonium acetate.

Reagents:
Neutral normal ammonium acetate solution (CH_3COONH_4): 77.08 g of ammonium acetate was dissolved in distilled water and made up to 1L. The pH was adjusted to 7 with acetic acid or ammonium hydroxide.

Procedure:
Extraction of available Calcium and Magnesium: 5 g of soil was shaken with 25 mL of neutral normal ammonium acetate for 5 minutes and filtered immediately through a dry Whatman No: 42 filter paper. First few mL of the filtrate was discarded.

Estimation of Calcium and magnesium by Atomic Absorption Spectrophotometry: From the soil extract, Ca and Mg can be estimated by Atomic Absorption Spectroscopy (AAS). The chemical interference, resulting from the formation of stable compounds between Ca and Mg ions and the accompanying anions may reduce the absorption. This interference may be overcome by using a realizing agent such as Lanthanum or Strontium.

Calculation:
Available Ca/Mg (mg kg^{-1} soil) = µg Ca/Mg mL^{-1} of the aliquot × 25/5

Available Ca/Mg (mg kg^{-1} soil) = µg Ca/Mg mL^{-1} of the aliquot × 5

Estimation of extractable Iron (Fe^{2+}, Fe^{3}), Manganese (Mn^{2+}), Zinc (Zn^{2+}), Copper (Cu^{2+}) and Chromium (Cr) (Lindsay & Norvell, 1978)

Principle: The major categories of micronutrient extract presently in use are dilute acids and solutions containing agents such as DTPA or EDTA.

Dilute acids (0.025-0.1 M) have been used as micronutrient extracts for many years, primarily on acidic soils. Their applicability is confirmed to acidic soils because they generally are not sufficiently buffered to extract meaningful levels of micronutrients from calcareous soils. Acidic extractants do not have a particularly sound theoretical basis, but due to their extensive use in field and laboratory studies, a well-developed database exists relating acid extractable levels of micronutrients to crop response. The most commonly used dilute acids are: Mehlich -1 (dilute double acid, 0.0125 M H_2SO_4 + 0.05 M HCl) (Sims & Johnson, 1991). Among the chelating agents, DTPA is the most commonly used one. The DTPA soil test, developed for near neutral and calcareous soil (Lindsay & Norvell, 1978) illustrates the evolution of the soil test extractant from theoretical principles. The extracting solution consisted of 0.005 M DTPA and 0.01 M $CaCl_2.2H_2O$, buffered at pH 7.3 by 0.1 M triethanolamine (TEA). The DTPA extractant offered the most favourable combination of stability constants necessary to simultaneously extract four micronutrient cations (Fe, Mn, Cu, Zn & Cr). The buffered pH and presence of soluble Ca^{2+} prevented excessive dissolution of calcium carbonate and avoided the release of available micronutrients occluded by this solid phase. At pH 7.3, 70-80 % of the buffering capacity provided by TEA was consumed. Therefore, use of DTPA extractant on acidic soils resulted in neutralization of remaining buffer capacity and unpredictable extraction pH.

Reagents:

Hydrochloric acid (HCl), 0.1 N: 8.1 mL of concentrated HCl (reagent grade) was added to approximately 900 mL of distilled water, mixed and cooled to room temperature and made up to 1litre.

DTPA: The extractant was prepared by dissolving 1.967 g of 14.92 g of TEA and 1.47 g of $CaCl_2.2H_2O$ in 200 mL of distilled water and diluted to approximately 900 ml. The pH was adjusted to + or - 0.05 with 1:1 HCl and made up the volume to 1 litre.

Extraction and estimation:

2 g of soil was shaken with 20 mL of 0.1 M HCl for 5 minutes. It was filtered through Whatman No.42 filter paper. The filtrate was collected and the contents of Fe, Mn, Zn, Cu, Cr estimated using an Atomic Absorption Spectrophotometer.

Calculation

$$\text{Amount of micronutrient (mg kg}^{-1}\text{ soil)} = \text{Concentration from the instrument} \times \frac{20}{2}$$

ie, Amount of micronutrient (mg kg^{-1} soil) = Concentration from the instrument x 10

Statistical Analysis

The mean value and standard deviation of various parameters in the study were determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSIONS

The results obtained on the uptake of various essential mineral nutrients and the non-essential metal Cr^{VI} by *Amaramthus dubius* in response to different concentrations of hexavalent chromium (Cr^{VI}) treatments indicate variations induced by the treatments over the control (table 6.1).

Table 6.1: The impact of Cr^{VI} stress on the content of various nutrient elements in *Amaranthus dubius* plant at 45th day after treatment

Treatm-ents- Cr^{VI}	Content of nutrient elements in the shoot component of *Amaranthus dubius*									
	Macronutrients					Micronutrients				Heavy metal
	N	P	K	Ca	Mg	Zn	Cu	Mn	Fe	Cr
0mg (Control)	7.570 ± 0.31	0.310 ± 0.06	0.093 ± 0.02	3.300 ± 0.11	5.947 ± 0.08	.0008 ± 0.0001	.0001 ± 0.0000	.0053 ± 0.0012	2.0867 ± 0.070	0.0005 ± 0.0002
1mg	7.297 ± 0.28	0.287 ± 0. 04	0.103 ± 0.02	3.383 ± 0.14	5.860 ± 0.11	.0013 ± 0.0006	.0001 ± 0.0000	.0070 ± 0.0017	2.1033 ± 0.102	0.0009 ± 0.0001
5mg	6.563 ± 0.19	0.247 ± 0.04	0.083 ± 0.02	2.967 ± 0.15	5.737 ± 0.07	.0107 ± 0.0012	.0001 ± 0.0000	.0093 ± 0.0006	2.2100 ± 0.098	0.0033 ± 0.0023
10mg	6.437 ± 0.11	0.240 ± 0.03	0.077 ± 0.02	2.927 ± 0.16	5.547 ± 0.48	.0117 ± 0.0012	.0001 ± 0.0000	.0107 ± 0.0015	3.0800 ± 0.0272	0.0067 ± 0.0012
20mg	5.520 ± 0.14	0.193 ± 0.03	0.067 ± 0.01	2.217 ± 0.11	5.367 ± 0.14	.0137 ± 0.0021	.0001 ± 0.0000	.0113 ± 0.0012	3.1133 ± 0.155	0.0097 ± 0.0015
30mg	5.233 ± 0.06	0.183 ± 0.03	0.063 ± 0.01	2.063 ± 0.11	5.360 ± 0.05	.0057 ± 0.0015	.0001 ± 0.0000	.0343 ± 0.0482	1.7700 ± 0.741	0.0113 ± 0.0021
50mg	4.830 ± 0.24	0.170 ± 0.01	0.057 ± 0. .01	1.757 ± 0.59	5.067 ± 0.17	.0023 ± 0.0015	.0001 ± 0.0000	.0053 ± 0.0012	1.5100 ± 0.529	0.0117 ± 0.0015
70mg	4.367 ± 0.19	0.167 ± 0.02	0.057 ± 0.01	1.633 ± 0.56	4.963 ± 0.16	.0016 ± 0.0012	.0001 ± 0.0000	.0047 ± 0.0012	1.2133 ± 0.137	0.0123 ± 0.0012

All the Cr^{VI} treatments caused more or less decrease in nitrogen content over the control. The lowest Cr^{VI} stress of 1mg Cr^{VI}/kg soil treatment recorded lowest reduction which is insignificant ($P<0.05$) over the control. Further enhancement in the Cr^{VI} stress caused gradual increase in the reduction of N content and the maximum reduction is recorded in the highest Cr^{VI} stress of 70mg Cr^{VI}. With the exception of initial level stress of 1mg

Cr^{VI}, all the stress levels have induced significant reduction in N content over the control. The content of other macronutrients like phosphorus (P), potassium (K), calcium and magnesium elements also recorded gradual reduction along with the increase in Cr^{VI} stress, but with the exceptions with respect K and Ca elements in the initial level Cr^{VI} stress of 1mg, which recorded an increase over the content of control (Figure 6.1).

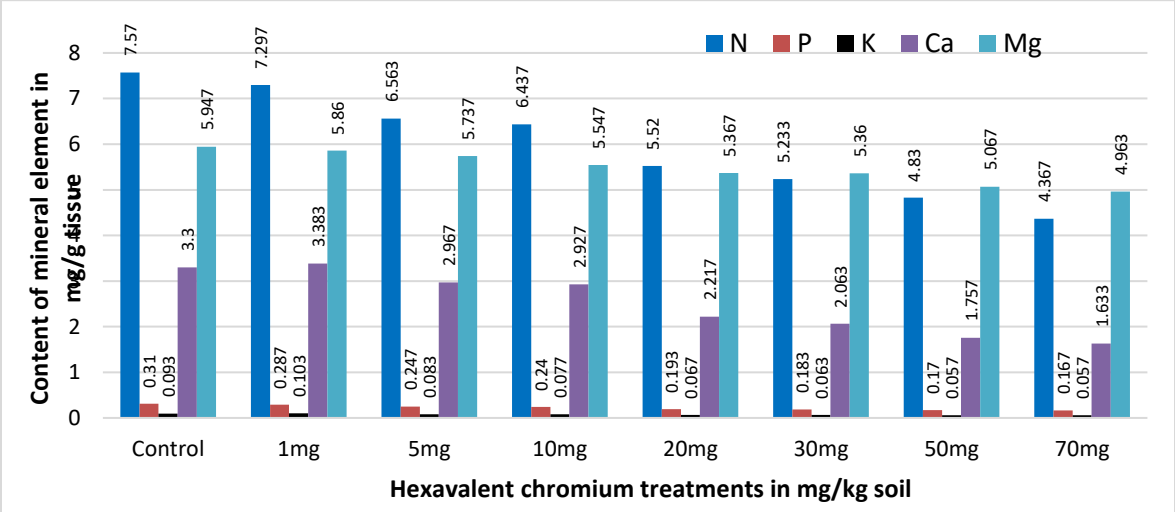

Figure 6.1: Impact of Cr^{VI} stress on the content of macronutrient elements

The statistical analysis revealed that, with the exception of initial level stress of 1mg Cr^{VI} with respect to N & P elements; 1mg & 5mg Cr^{VI} with respect to Ca & Mg and 1mg, 5mg & 10mg Cr^{VI} respect to K element, the reduction in the content caused by all the other Cr^{VI} stress treatments is significant over the control (table 6.2, 6.3, 6.4, 6.5 and 6.6).

Table 6.2: Duncan analysis of nitrogen (N) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	4.3667				
7 (50mg)		4.8300			
6 (30mg)			5.2333		
5 (20mg)			5.5200		
4 (10mg)				6.4367	
3 (5mg)				6.5633	
2 (1mg)					7.2967
1 (Control)					7.5700
Sig.	1.000	1.000	.108	.463	.124

Means for groups in homogeneous subsets are displayed.

Table 6.3: Duncan analysis of phosphorous (P) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	.1667			
7	.1700			
6	.1833	.1833		
5	.1933	.1933		
4		.2400	.2400	
3		.2467	.2467	
2			.2867	.2867
1				.3100
Sig.	.400	.055	.138	.423

Means for groups in homogeneous subsets are displayed.

Table 6.4: Duncan analysis of Calcium (Ca) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8	1.6333				
7	1.7567	1.7567			
6		2.0633	2.0633		
5			2.2167		
4				2.9267	
3				2.9667	2.9667
1				3.3000	3.3000
2					3.3833
Sig.	.522	.123	.428	.078	.051

Means for groups in homogeneous subsets are displayed.

Table 6.5: Duncan analysis of Magnesium (Mg) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8	4.9633				
7	5.0667	5.0667			
6		5.3600	5.3600		
5		5.3667	5.3667		
4			5.5467	5.5467	
3			5.7367	5.7367	5.7367
2				5.8600	5.8600
1					5.9467
Sig.	.542	.104	.051	.091	.247

Means for groups in homogeneous subsets are displayed.

Table 6.6: Duncan analysis of potassium (K) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
7 (50mg)	.0567		
8 (70mg)	.0567		
6 (30mg)	.0633	.0633	
5 (20mg)	.0667	.0667	
4 (10mg)	.0767	.0767	.0767
3 (5mg)		.0833	.0833
1 (Control)			.0933
2 (1mg)			.1033
Sig.	.088	.083	.237

Means for groups in homogeneous subsets are displayed.

Nitrogen (N) is the most essential macronutrient element which support the growth and development of plants as it constitutes the important component of chlorophyll, amino acids, nucleic acids and protoplasm. The inference obtained in the study clearly revealed the metal Cr^{VI} is toxic and inhibitory in action against the uptake of N element and the degree of inhibition is dose dependent. Like nitrogen element, phosphorus (P) and potassium (K) elements are also considered as crucial macronutrient for plant growth and development. Phosphorous is a vital part of nuclear protein and ATP and important element for metabolism of sugars and photosynthesis, while potassium is indispensable for the opening and closing of stomata for various gaseous exchange activities, for stimulating the activities of many enzymes of protein synthesis, photosynthesis, nitrogen and carbon metabolism etc. With respect to the macro element magnesium (Mg^{2+}), which constitute the central core of chlorophyll molecule, its deficiency could negatively affect chlorophyll biosynthesis and rate of photosynthesis and this in turn may cause chlorosis and stunted plant growth. The macronutrient calcium (Ca^{2+}) performs the role like selective permeability of cell membrane, sustaining cell turgidity etc. The roles of these two elements clearly indicate the essentiality of these elements like other macro elements for the proper growth and development of plants.

It is generally understood that the occurrence of heavy metals, when exceeds the permissible level in the soil environment, affects the uptake of nutrient elements by the plant roots due to different reasons. The reduction in the content of N element inferred in

A. dubius under Cr^{VI} stress in the study may be the result of decreased uptake of N element due to damaged plasma membrane and excessive membrane leakage in the roots induced by Cr^{VI} toxicity (Huang & Xiong, 2009). Further, it might also be contributed by the less availability of available nitrogen in the soil due to disruption in the nitrification and ammonification processes through modulating the activities of nitrogen fixing bacteria under Cr^{VI} toxicity (Kapoor *et al*., 2015). The reduction in the content of phosphorus (P), potassium (K) and other macro elements in the shoot component of Cr^{VI} treated *A. dubius* plants over the control could be due to failure in the transport of these elements by the damaged cells of root under Cr^{VI} toxicity (Dahiya *et al*., 2003). The decline in the content of macronutrients N, P, K, Ca and Mg in the shoot component of *A. dubius* under excessive Cr^{VI} metal inferred in the study can be due to many other factors too. The disrupted uptake of essential macro elements resulting from masking the sorption sites by insoluble complexes formed by the metal Cr^{VI} can be one reason (Kabata & Szteke, 2015; Osu & Onyema, 2016). Further, Cr^{VI} toxicity induced poor growth and development of root system, impairment of the root penetration and reduced root surface area could have been the reason which led to the reduced nutrient uptake and translocation of these elements and corresponding decrease in their content in the shoot. Another major possible fact behind the decline may be the result of reduced translocation of these essential elements due to displacement by the metal Cr^{VI} from physiologically important binding sites (Shahzad *et al*., 2018; Mengel & Kirkby, 1987).

The uptake of micronutrients zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) under Cr^{VI} stress exhibits a different trend compared to macronutrients. The content of Fe & Zn in the shoot component showed a gradual increase over the control up to a stress of 20mg Cr^{VI}/kg soil and for Mn up to 30mg Cr^{VI}/kg soil treatment. But then onwards, further increase in the stress caused reduction in their content. The micronutrient Cu detected only in trace quantities in *A. dubius* plants treated with all the levels of Cr^{VI} stress as well as in the control, while with respect to the uptake and content of metal Cr, gradual and progressive increase recorded up to the highest stress of 70mg Cr^{VI}/kg soil treatment and revealed a dose dependent increase in their content (figure 6.2).

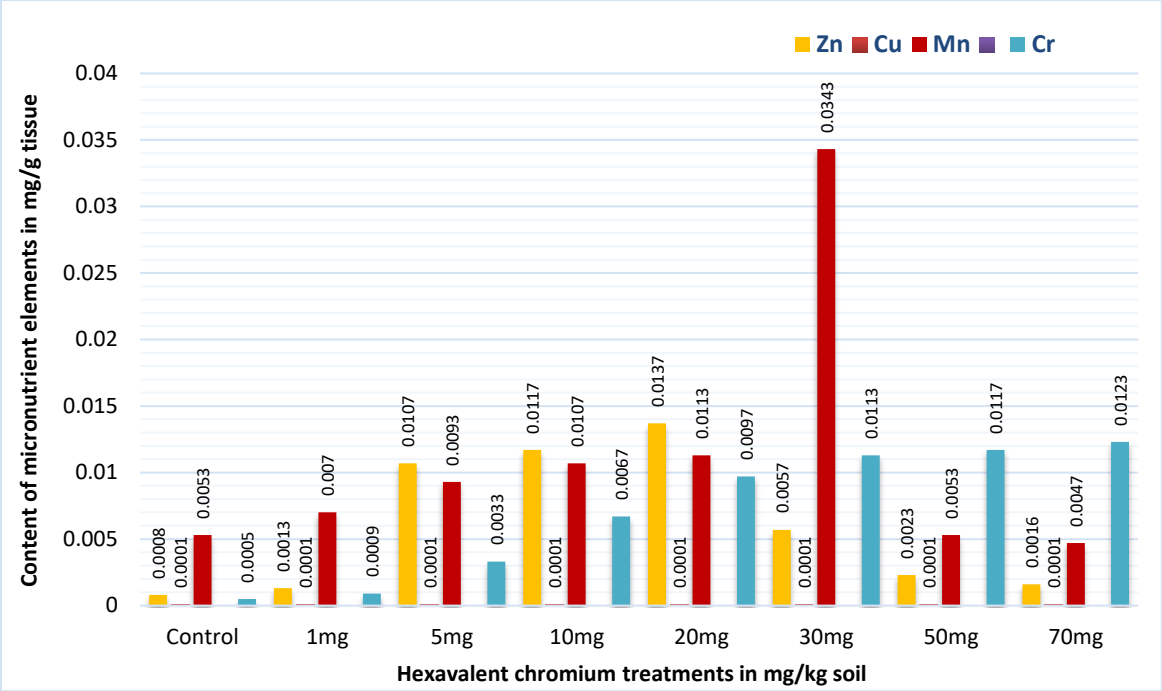

Figure 6.2: Impact of Cr^{VI} stress on the content of micronutrients Zn, Cu, Mn and the metal Cr^{VI} in *A. dubius*

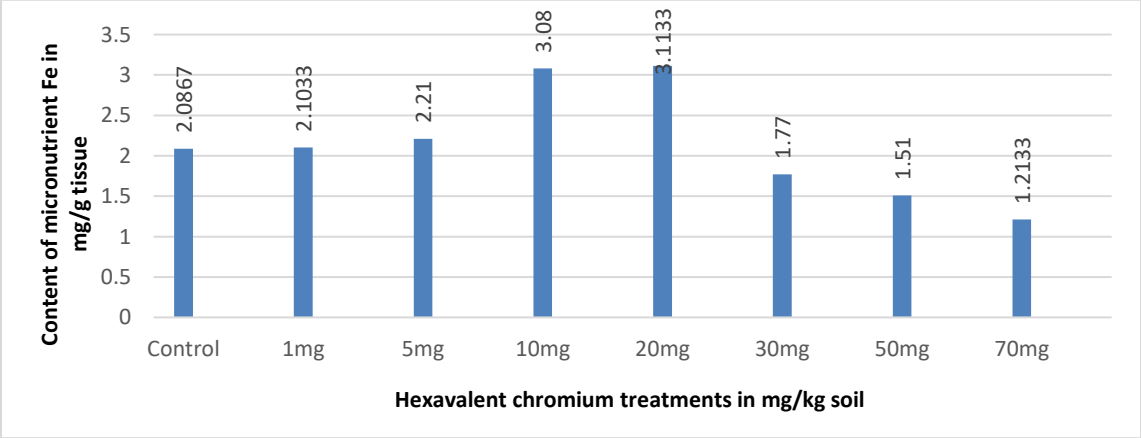

Figure 6.3: Impact of Cr^{VI} stress on the content of micronutrient Fe in *A. dubius*

The analysis of changes in micronutrients content revealed the increase in the content of Zinc (Zn) due to initial level stress of 1mg Cr^{VI} and higher levels of 50mg & 70mgCr^{VI} are insignificant over the control while others are significant ($P<0.05$) (table 6.7). With respect to the iron (Fe) content, the variations due to 1mg & 5mgCr^{VI} stress are insignificant increase while 30mg & 50mg Cr^{VI} are insignificant decrease over the control, whereas the increases in the content due to10mg & 20mgCr^{VI} stress and the

decrease due to 70mg Cr^{VI} stress over the control are significant (table 6.8 & figure 6.3). The variations in the content of manganese (Mn) induced by Cr^{VI} stress are found insignificant over the control in all the treatment levels (table 6.9). With respect to the content of non-essential metal chromium (Cr^{VI}), with the exception of 1mgCr^{VI} stress, the increase in the content caused by all the hexavalent chromium treatments is significant (table 6.10).

Table 6.7: Duncan analysis of Zinc (Zn) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
1 (Control)	.0008			
2 (1mg)	.0013			
8 (70mg)	.0016			
7 (50mg)	.0023			
6 (30mg)		.0057		
3 (5mg)			.0107	
4 (10mg)			.0117	.0117
5 (20mg)				.0137
Sig.	.200	1.000	.358	.077

Means for groups in homogeneous subsets are displayed.

Table 6.8: Duncan analysis of iron (Fe) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	1.2133			
7	1.5100	1.5100		
6	1.7700	1.7700	1.7700	
1		2.0867	2.0867	
2		2.1033	2.1033	
3			2.2100	
4				3.0800
5				3.1133
	.081	.072	.173	.908

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8	1.2133			
7	1.5100	1.5100		
6	1.7700	1.7700	1.7700	
1		2.0867	2.0867	
2		2.1033	2.1033	
3			2.2100	
4				3.0800
5				3.1133
	.081	.072	.173	.908

Means for groups in homogeneous subsets are displayed.

Table 6.9: *Duncan analysis of manganese (Mn) content in A. dubius plants treated with* Cr^{VI}

Treatment Code Cr^{VI} */ kg soil*	*Subset for alpha = 0.05*
	1
8	.0047
1	.0053
7	.0053
2	.0070
3	.0093
4	.0107
5	.0113
6	.0343
Sig.	.079

Means for groups in homogeneous subsets are displayed.

Table 6.10: Duncan analysis of chromium (Cr^{VI}) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
1 (Control)	.0005			
2 (1mg)	.0009	.0009		
3 (5mg)		.0033		
4 (10mg)			.0067	
5 (20mg)				.0097
6 (30mg)				.0113
7 (50mg)				.0117
8 (70mg)				.0123
Sig.	.701	.061	1.000	.055

Means for groups in homogeneous subsets are displayed.

According to the Hard–Soft Acid–Base (HSAB) theory put forward by Pearson, certain non-essential heavy metals like Cr, Pb, Cd etc. have the similar properties of some essential nutrient elements like Fe, Mn, Zn, Cu, Mg, P, Ca, K etc. (Housecroft & Sharpe, 2005). Bridges and Zalups in 2005 reported disrupted uptake of Fe, Mn, Zn and Cu by the plants growing in areas where the metal Cr concentration exceeds permissible level. According to him the metal Cr has similarity to the chemical as well as physical properties of these essential metals and this resulted in competition between Cr and these essential ions for binding sites of metal absorptive and enzymatic proteins and this in turn reduced content of these essential nutrients in such plants. A similar observation of reduced intake of micronutrients like Zn, Cu, Fe and Mn is reported by Sundaramoorthy *et al*. (2010) in paddy plants under Cr stress and similarly, several other reports are also available in support of the disrupting effect of metal chromium on the uptake of essential elements such as Mn, Cu, Zn, Fe, Ca and Mg (Turner & Rust, 1971; Moral *et al*., 1995; Chatterjee & Chatterjee, 2000; Gardea *et al*., 2004; Zeng *et al*., 2010). The significant reduction recorded in the content of micronutrients like Fe, Mn and Zn at very higher Cr^{VI} stress treatment of 30mg, 50mg & 70mg Cr^{VI}/kg soil over the control in the present study are in agreement with the above observations and reports. The very high concentration of Cr^{VI} in the growing media might have led to the increased competition and exchangeability between the Cr^{VI} and these ions (Eller & Brix, 2016). This in turn may lead to a regulatory control over these elements which might be the reason for reduction in their content (Chaney, 1978; Swamy, 1998). However, at the same time *A. dubius* plants treated with comparatively lower-level concentrations of 1mg, 5mg, 10mg & 20mg Cr^{VI}/kg soil treatment recorded a contradictory result, where the content of Fe, Mn and Zn progressively increased over the control treatment. This inference in the study is in agreement with the observations of enhanced uptake and content of nutrient iron (Fe) in *Veronica* plant species (Zivkovi´c *et al*., 2012); increased uptake and content of manganese (Mn) in *Citrullus vulgaris* (Dube *et al*., 2003) and similar observation in *Lolium perenne* (Vernay *et al*., 2007) under chromium stress. Besides this observation on micronutrients, the study also observed previously an insignificant increase in the content of macronutrient calcium (Ca) and potassium (K) over the control, although it is only observed in the initial level stress of 1mg Cr^{VI}/kg soil treatment. The increase in the

content of said micro and macro elements observed under Cr^{VI} stress may be a part of mechanism of the plant to impart tolerance to Cr^{VI} toxicity by enhancing the activity of these nutrients' dependent antioxidant enzymes (Yang & Poovaiah, 2003; Millaleo *et al*., 2010; Fang *et al*., 2014).

CONCLUSION

The results obtained in the study revealed the non-essential hexavalent chromium (Cr^{VI}) stress in the growing medium affected the uptake and accumulation of both macro and micro nutrient elements in *Amaranthus dubius*. The major reason that led to the disrupted uptake of essential nutrients in the study could be the result of Cr^{VI} toxicity induced poor root growth and reduced surface area, impaired root penetration and damage to the root system. This is evident from the inferences obtained from the root morphological and anatomical growth parameters analysed in response to Cr^{VI} stress in the chapter 2 and chapter 6 respectively. The excess occurrence of metal Cr^{VI} in the growing environment and this oriented higher competition and exchangeability between the Cr^{VI} ions and essential nutrient ions due to their similarities, during the absorption by the roots could be another major reason. The masking the sorption sites in root by insoluble complexes formed by the metal Cr^{VI} and as well as the displacement of essential nutrients from physiologically important binding sites by the metal can be another reason that contributed towards the reduction in the content of essential nutrient elements in the shoot component of *A. dubius*. The study concludes that, the excess concentration of metal Cr^{VI} in the growing environment is toxic to the leafy vegetable *A. dubius*, causing disruption in the uptake of essential nutrient elements and their corresponding translocation to the shoot component, which are necessary to carryout various physiological and metabolic growth processes. Therefore, the study in general suggested the existence of a negative correlation between the level of Cr^{VI} stress and the availability of essential nutrients as well as the growth performance in *A. dubius*.

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CHAPTER-7

IMPACT OF HEXAVALENT CHROMIUM (Cr^{VI}) STRESS ON NUTRITIONAL QUALITY

INTRODUCTION

Human diet mainly comprises of edible crops among which green leafy vegetables are identified to be abundant in dietary fibre, vitamins, fatty acids, amino acids etc. It is a known fact that vitamins and both macro and micro nutrients are vital for good health and proper functioning of immune system. Vitamins that are available in food products in complex organic compound groups protect the tissues from oxidative impairment (Finger *et al*., 2009; Shimizu *et al*., 2018), maintain the good health of nervous and cardiovascular systems, brain and bones (Combs, 2007; Atkins *et al*., 2009), engage in metabolism of carbohydrates, detoxify the liver, take part in erythrocytes synthesis etc. (Crider *et al*., 2011). Another indispensable nutrient for our wellbeing is the fatty acid which can be made available only through dietary food intake as it cannot be synthesized in the body. Alpha linolenic acid (ALA) is the fatty acid in plants and is mainly available in the chloroplast of green leafy vegetables (Kaur *et al*., 2014). Fatty acids regulate the immune and central nervous system, maintains the health of heart and skin and prevents cancer and obesity etc. (Kaur *et al*., 2014). Amino acid lysine is another main micronutrient needed for good human health which is also to be made available through proper diet. Lysine controls wound healing (Guo & DiPietro, 2010), protects cold sore caused by herpes simplex virus HSV-1(Griffith *et al*., 1981), absorbs and retends the calcium in the body (Civitelli *et al*., 1992) etc. Unlike fats and carbohydrates, the dietary fibre has not caught much attention as a micronutrient. Dietary fibre comprises of carbohydrates in non-digestible form, generally as polysaccharides that are produced from leafy vegetables, fruits etc. The consumption of dietary fibre boosts gut motility and prevents constipation (Rao *et al*., 2015), improves insulin responsiveness and metabolic wellbeing (Weickert *et al*., 2006), lower the chance of cardiovascular mortality and ailments (Kim & Je, 2016), aid in weight loss by reducing food consumption etc. (Solah *et al*., 2017).

The nutritional value of any crop is influenced by the physical, chemical as well as biological environment in which it grows. Environmental contamination arising from the enhancing industrialization in the recent year's gain relevance in this context. The nutritional value of crop plants gets deteriorated considerably when grown in extreme environmental conditions of increased heavy metal concentrations, pH and temperature (Ipek *et al*., 2005). It is observed that the nutritional quality of vegetable crops grown in soil applied with inorganic fertilizers, pesticides, herbicides, fungicides etc. and irrigated using waste water and industrial pollutants is very low with significant variations in comparison with those grown in organic environment having normal water supply. The main cause for this may be the oxidative stress due to the presence of heavy metals in excess concentrations. The oxidative damage induced by heavy metals can lead to the degrading of chloroplast and peroxidation of lipids (Khanna-Chopra, 2012) which may cause a negative effect on the functioning and content structure of nutrients and vitamins in crops (Sugawara *et al*. 1981; Pleasants *et al*. 1992). Human health is affected by heavy metals, both directly and indirectly, and the indirect effect is caused by the consumption of degraded vegetables with varied nutritional quality grown in heavy metal stressed environment. Metal toxicity and nutritional deficiency are the results of consuming such vegetables which may cause serious health issues. Due to this reason, heavy metal contamination in agricultural soil is a grave issue to be addressed. As of today, the available literature indicates that the research on adverse effects of heavy metals and other effluents on vegetative crops are concentrated on bioaccumulation and their translocation, physiology and metabolism correlated to growth and productivity etc. Little information and awareness are available on the effect of heavy metals on the nutritional constitution of vegetable crops. The present study gains relevance in the context that there has been no scientific analysis conducted so far on the impact of hexavalent chromium (Cr^{VI}) stress on the nutritional constituents like vitamins, fatty acids, dietary fibre content, amino acids etc. of *Amaranthus dubius* (CO-1).

REVIEW OF LITERATURE

Basically, vegetables are termed protective foods owing to their capability to resist diseases and hence consumed more considering the presence of many nutritionally vital compounds mandatory for human survival (Heiner *et al*., 2012). Contamination of soil, water and air by heavy metals has a serious impact on the quality of food we eat and thereby on our health. The consumers of metal contaminated vegetables are prone to a variety of diseases. The main pathway for entry of heavy metals into human body is through the intake of contaminated vegetables (Nriagu, 1990). The heavy metals influence the nutritional components of plants depending on the type of food crop, soil environment, exposure time and plant organs (Gonçalve *et al*., 2009). Nordberg (1996) and Fox (1988) observed that the population consuming metal polluted food have a deficiency of both macro and micro nutrients such as vitamins, minerals like Ca, Fe and Zn, fats and proteins. Several physiological and pathological ailments are identified to develop due to exhaustion of nutrients like Fe and vitamins on consumption of metal adulterated food (Iyengar & Nair, 2000). Excessive concentration of heavy metals entering the food chain and their consumption may result in depletion of important nutrients causing grave health issues (Arora *et al*., 2008; US Department of health and human services, 2005).

Vitamins have the capability to retard oxidative stress and synchronize the immune system thereby preventing a number of chronic ailments like cardiovascular disease, cancer etc. (Haard, 1984; Monsen, 2000). Studies indicate that the measure of plant antioxidants vary on exposure to metal components (Collin *et al*., 2008; Hou *et al*., 2018). The functions and contents of vitamins in plants are negatively affected when subjected to heavy metals (Pleasants *et al*., 1992). A negative correlation is noticed between heavy metal content and vitamins via when the concentration of heavy metal increases, the vitamin contents reduce and vice versa (Widowati, 2012; Munzuroglu *et al*., 2005). All vitamins are essential for our health. Vit A is needed for good vision, growth and development of cells, improvement of antioxidant enzyme functions (Malivindi *et al*., 2018) and brings down the level of oxidative stress (Shimizu *et al*.,

2018); Vit K is required for blood clotting (Shearer *et al*., 2012, Shearer & Newman, 2014) and maintains good bone health (Atkins *et al*., 2009). Vit.B6 carries out the functions of producing red blood cells, detoxification of liver, maintains brains and nerves in good health and regulates carbohydrate metabolism (Combs, 2007) while Vit.B3 alternately called niacin basically produces energy from carbohydrates, dietary proteins and fats (Leskova *et al*., 2006). Enzymes contained in Vit.B3 act as scavengers of free radicals and safeguard tissues from oxidative damage (Finger *et al*., 2009). Vit. B9 known as folate is essential for brain health and supports cardiovascular and nervous system in human beings and is necessary for the synthesis of erythrocytes; more intake of folates minimize the risk of breast cancer in females (Crider *et al*., 2011).

There are contradictory reports on variations in the concentrations of amino acids and proteins induced by heavy metals. In some plant species (*Brassica juncea* and poplar), a decrease is noticed in many proteins and amino acids whereas they show an increasing tendency in some other plants like *Arabidopsis thaliana* (Alvarez *et al*., 2009; Sarry *et al*., 2006). At high levels of Cd, a decrease in protein content is observed in wild carrot (*Daucus carota*) and common sunflower (*Helianthus annuus*) as identified by Lin *et al*. (2007) and Mahmood *et al*. (2005). Degradation of protein accelerates with enhanced protease activity (Xu *et al*., 2006) or intervention with nitrogen metabolism due to the presence of heavy metals, causing decrease in the content of proteins. Abou and Ali (2010) argued that heavy metals are capable of disturbing nitrogen metabolism that may decrease protein synthesis in vegetables and are accountable for reduced photosynthesis leading to reduced protein synthesis (Lin *et al*., 2007). Heavy metal stress has a remarkable adverse impact on the composition of dietary fiber, amino acids and proteins, moisture etc. of *Spinacia oleracea* and as reported by Alia *et al*. (2015), excessive stress of either cadmium or lead may cause a decrease in the nutritional contents by 22 to 33% and under the consolidated stress by both the metals, this decrease may evhen go up to 36 to 43% in comparison to control.

A remarkable system of oxidative strain in plants is the peroxidation of unsaturated fatty acid (Djebali *et al*., 2005). Lipid peroxidation occurs at higher levels of heavy metal

stress (Monteiro *et al*., 2004), which is produced by lipoxygenase activity induced by metals causing metal ion displacement and alteration in the functions of enzymes associated with biosynthetic pathways of fatty acids (Wildner & Henkel, 1979). Numerous aliphatic compounds like membrane glycerolipids, cutin/suberin, TAG, jasmonates, and nitroalkenes (NO_2-Fas) are produced in plants utilizing unsaturated fatty acids (C18 UFAs) as raw material and these compounds act defensive against many biotic and abiotic stresses thereby lowering UFA content to ease the oxidative damage induced by stress (Mei & Nai, 2020). Guédard *et al*. (2008) reported that in plants grown in heavy metal polluted soil, the concentrations of tri-unsaturated fatty acids (C18:3) is found to decrease while the concentrations of other fatty acids (C18:0, C18:1, and C18:2) is noted to increase. Therefore, fatty acids can act as bioindicators of heavy metal contamination in plants. Heavy metals manifest variable effects on different types of fatty acids and as understood by Djebali *et al*. (2005) and Ben *et al*. (2005), they have a negative influence on C18:3 percentages whereas other fatty acids experienced a positive impact.

Being the first structure of a plant cell, the cell membrane is the target of oxidative stress. The characteristics of the membranes depend on the composition of fatty acid and any change in its profile may bring about unusual process. Stress due to heavy metals have a decreasing impact on the level of unsaturated fatty acids (USFA) and an increasing effect on saturated fatty acids (SFA). In case of *Populous nigra* grown in heavy metal contaminated soil rich in Cd, Cr, Cu, Ni, Pb and Zn, the level of linolenic acid (18:3) considerably reduced and the content of linoleic (C18:2), oleic (C18:1) and stearic (C18:0) acid elevated in the leaves (Le Guedard *et al*., 2012). In sunflower plants cultivated in Cd stressed environment, the level of linolenic, oleic and linoleic acid considerably decreased, and the percentage of the palmitic significantly increased (Moradkhani *et al*., 2012). The amount of linolenic acid (18:3) and hexadecatrienoic acid (16:3) reduced while oleic acid (18:1) palmitic acid (16:0) and linoleic acid (18:2) shot up in the leaves of tomato plants cultivated in metal contaminated soils. On exposure of wheat seedling to heavy metal stress, linolenic acid (18:3) in shoots and roots of plants showed a decreasing tendency (Gajewska *et al*., 2012). The direct reaction of free

radicals with unsaturated fatty acids may be the cause for reduction in PUFA concentration like linoleic acid and palmitoleic acid (Dursun & Lokman, 2019). Alia *et al*. (2015) has reported the critical adverse effects of heavy metal stress on the fiber content of *Spinacia oleracea*. It is observed that there is a reduction of 29% fiber content at higher dosage of Cadmium over the control plants whereas a combination of Cadmium and Lead resulted in 37% reduction in *Spinacia oleracea* (Alia *et al*., 2015).

MATERIALS AND METHODS

The assessment the nutritional quality of *Amaranthus dubius* plants subjected to different concentration levels of hexavalent chromium (Cr^{VI}) stress involved the following nutrient component parameters.

a) Estimation of Vitamin A / Retinoic acid (Neeld & Pearson, 1963)

Standard Vitamin A Solution:

About 5 mg of all-trans vitamin A acetate was weighed and dissolved in 100 ml of chloroform and stored in an amber colored bottle at -10° C.

Trichloroacetic Acid Reagent:

30% Trichloroacetic Acid (TCA) solution was prepared by dissolving in chloroform and stored in a glass stoppered amber coloured bottle at refrigeration temperature. Before use, the reagent was warmed to room temperature (25^{o}C) and an appreciable amount transferred to a repipet dispenser bottle.

Dichloro-2-Propanol (1, 3-DCP):

The Dichloro-2-Propanol reagent was stored in an amber-coloured bottle at room temperature. Before use, the reagent was warmed to a temperature of 25^{o}C.

Colorimetric Determination

The wavelength of maximum absorption for the reagents 1, 3- DCP and Trichloroacetic Acid (TCA) in chloroform was analysed using known concentrations of vitamin A acetate in chloroform. The relationship between concentration and absorbance at 616 and 620nm were noted and used for estimation of vitamin A in the sample extract.

Sample Analysis

After separation an aliquot (1.0 ml) of the solvent extract was carefully pipetted with an autopipet into a 1-cm quartz cell. To this was added 1 ml of TCA in either chloroform or methylene chloride from a fast delivery pipette with maximum absorbance recorded at full scale deflection (usually within 5-7 seconds) at 620 and 616nm at different analysis times (within each day of analysis). All samples were run in duplicates within each day of analysis. Furthermore, 1, 3-dichlor-2- propanol (1, 3-DCP) activated with SbCl3 practical grade was utilized in sample analysis.

Calculation of Results:

Quantitative results were calculated from standard calibration graphs with reference to the reagent-solvent in question. Standard calibration graphs were made for each day before quantitative analyses were carried out.

b) Estimation of Vitamin K (Vire *et al.*, 1977)

Reagents

All of the sulfuric acid solvent solutions were made by dilution of concentrated sulfuric acid. The titanium (III) stock solutions were 15% $Ti_2(S0_4)_3$ in 23% H_2S0_4 by weight and the stock titanium (IV) was prepared by dissolving $TiCl_4$ in water.

The stock 2-methyl-1,4-naphthoquinone solutions were prepared by dissolving 100mg of naphthoquinone in 100mL of methanol as other compounds except the sodium 1,2-naphthoquinone-4-sulfonate, which was dissolved in water. These solutions were maintained in darkness to avoid decomposition.

An aliquot of each Vitamin K stock solutions (Menadione, 1,4-Naphthoquinone, Sodium 1,2-Naphthoquinone-4-sulfonate, 2-Methyl-3-phytyl-1,4-naphthoquin) were placed into a 25-mL volumetric flask and 0.5ml of the $Ti_2(S04)_3$ stock solution and 0.3ml M of the $TiCl_4$ stock solution (diluted twice with methanol to minimize the violence of the HCl evolution when mixed with 14 M H_2S0_4) were then added. After cooling, the volume of the solution was brought to 25ml with 14 M H_2S0_4 and left in the dark for 30 min prior to use.

All spectra were taken with a UV spectrophotometer at room temperature and blanks of identical solvent composition were employed in the reference beam.

c) Estimation of Vitamin B6 / Pyridoxine (Raza *et al.*, 2007)

Reagents

The ferric ammonium sulfate solution (NH_4 Fe $(SO_4)_2.12H_2O$) in distilled water containing 3ml of concentrated H_2SO_4 was prepared in 100ml volumetric flask.

Buffer solution (pH-3) was prepared by mixing of 22.3 ml of 0.1M HCl with 50 ml of 0.1 M potassium hydrogen phthalate and diluted to 100 ml by distilled water in a volumetric flask.

Preparation of plant extracts

1g plant extract was weighed and grounded. In order to extract vitamin B6, the plant material was treated with ammonium acetate/methanol 50:50(v/v) and then filtered through Whatman filter paper. The obtained solution was centrifugated for 15 min at 1500 rpm. The supernatant was recuperated and diluted to 50 ml volumetric flask by acetic acid.

Spectrophotometric method

In order to obtain standard curve of ferric ion the general procedure was applied: aliquots of standard pyridoxine hydrochloride solution equivalent 50-700µg (0.5ml-7ml) were transferred into a series of 25ml volumetric flasks, 0.5ml of buffer solution pH 3, and 7ml of ferric ammonium sulfate solution were added. The content is mixed and then diluted to the mark with distilled water and mixed well. The absorbance was measured at 465nm against a blank reagent.

d) Estimation of Vitamin B3 / Niacin (Ranganath & Chowdary, 2014)

Selection of common Solvent

Methanol: water in the ratio of (40:60 v/v) solution was selected as a common solvent

Preparation of standard solution

The standard stock solutions of niacin were prepared by dissolving 100 mg of drug in 100ml volumetric flask, then sonicated for 30 min and the final volume was adjusted with the solvent to obtain a concentration of 1000 µg/ml of drug (stock-I solution).

A standard solution of 20 µg/ml of niacin was prepared by diluting 1ml of standard stock-I solution to 50ml with the solvent and the drug solutions were scanned in the entire UV range to determine the absorption maxima (λmax) of the drugs. The absorption maxima of niacin were found to be 262 nm and 250 nm as isosbestic absorption wavelength. A series of standard solutions were prepared by taking aliquots of stock-I and diluted to

obtain the concentration in the range of 10–50 µg/ml for niacin. The absorbances of resulting solutions were measured at 246 nm, 250 nm and 262 nm. The absorbance values Vs concentrations were used to plot the calibration curves. Drug obeyed linearity in the above concentration range.

Veroirdt's method (Simultaneous Equation Method)

This method is based on the absorption of niacin at the maximum wavelength. The wavelength selected for the development of simultaneous equations was 262 nm which is the absorption maxima (λmax) of niacin. The absorbance of niacin was measured at the selected wavelengths. The absorptive values were determined for the drug at the selected wavelengths. These values were the mean of five estimations.

The concentration of the drug in mixture can be calculated by using the following equations: -

$CN = A_1 – A_2 / ax_1 – ax_2$

Were,

CN = concentration of niacin

A1 = absorbance of mixture at 262 nm

A2 = absorbance of mixture at 246 nm

ax1 = absorptivity of niacin at 262 nm

ax2 = absorptivity of niacin at 246 nm

e) Estimation of Vitamin B9 / Folic Acid (Hulchings *et al*., 1947)

Principle

Folic acid is extracted from samples using mild alkaline buffer, oxidized with permanganate, and the resulting amine is diazotized. The diazotized compound is coupled with N-(1-naphthyl) ethylenediamine, and the colour developed is determined at 550 nm.

Reagents

1. Dibasic potassium phosphate solution: 60.61 g K_2HPO_4 is dissolved in water and made up to 2 litres.

2. Potassium permanganate solution: 0.4g of $KMnO_4$ was taken in a 100- ml volumetric flask and diluted to volume with water.

3. Sodium nitrite solution: 2g of $NaNO_2$ was taken in a 100-ml volumetric flask and made up to volume with water.

4. 5 N HCL

5. Ammonium sulphamate solution: 5g (NH_4) NH_2SO_3 was dissolved in water and made up the volume to 100 ml.

6. N-(1-naphthyl) ethylenediamine dihydrochloride solution: 0.1 g of the substance is placed in a 100 –ml volumetric flask and diluted to volume with water.

7. Sodium chloride

8. iso- Butyl alcohol

9. Stock folic acid standard solution: 50 mg of folic acid is dissolved in water with the help of 2 ml of NH_3, and made up the volume to 100ml with water (1ml = 500µg)

10. Working folic acid standard solution: An aliquot of the stock standard solution was diluted with K_2HPO_4 solution (Reagent1) to give a concentration of 10 µg/ml.

Assay

A known amount of the sample containing about 100mg of folic acid was placed in a 100-ml flask. About 50 ml of K_2HPO_4 solution was added and the mixture was heated to a temperature not above 60^0C with swirling until the sample was properly dispersed. Cooled to room temperature and made up the volume to 100 ml with K_2HPO_4 solution. The solution was centrifuged and transfered an aliquot of the clear solution containing about 1 mg of folic acid to a 100-ml volumetric flask. Diluted to volume with K_2HPO_4 solution. Used this solution for color development and estimation.

Preparation of duplicate assay tubes as follows:

Tube No	Sample (ml)	Working Std. (ml)	Water (ml)	Potassium permanganate solution (ml)
1	5.0	0.0	0.0	1.0
2	5.0	2.0	0.0	1.0
3	5.0	0.0	1.0	0.0
4	5.0	2.0	1.0	0.0

1ml sodium nitrite solution and 1ml of 5 N HCL was added to all the tubes. Mixed and allowed standing for 2 minutes. 1ml of ammonium sulphamate solution was added and mixed with swirling. 1ml of N-(1-naphthyl) ethylenediamine dihydrochloride solution was added, mixed and allowed to stand for 10 min. 1g of sodium chloride and 10 ml of *iso*-butyl alcohol was added. Shaked vigorously for 2-3min. The *iso*-butyl alcohol was seperated by centrifugation and removed about 9 ml of the clear supernatant layer. The color of the *iso*-butyl alcohol was read at 550 nm within 25 min using *iso*-butyl alcohol as the blank.

Calculate the quantity of folic acid in the sample preparation in mg/ml using the formula:

$$0.4\ C = \frac{A1-A2}{A2 + A3 - (A1 + A4)}$$

Where C = concentration of the working standard of folic acid in mg/ml, and A1, A2, A3and A4 are the absorbance tubes 1,2,3 and 4 respectively.

f) Estimation of Lysine (Ferrel *et al.*, 1968)

Reagents and Equipments

Stock solutions of lysine HCl containing 5mg/ml was stored under refrigeration and working standards were prepared by dilution, as required.

The ninhydrin reagent was prepared by dissolving 0.5g ninhydrin (1,2,3-triketohydrindene) and 26.5g sodium chloride in about 70 ml water plus 10 ml 0.100N sodium hydroxide and diluted to 100ml.This reagent is stable for several weeks at 4ºC.

Colorimetric measurements were made in a UV Spectrophotometer using matched square cuvets with a 13mm light path.

Assay

1. The sample material 0.5 to 5.0g was weighed depending on expected amino acid content. The material was grounded to pass a 40- mesh sieve and transfered to a 100 ml volumetric flask. A similar sized sample of unfortified material was weighed at the same time to be run in parallel, as a sample blank to correct for naturally occurring ninhydrin reaction materials.
2. 50 ml of 0.1N hydrochloric acid was added with care to assure uniform sample dispersion and extracted with the help of mechanical shaking for 15 minutes
3. Diluted to volume with distilled water and filtered through a moderately fast paper.
4. Diluted an aliquot of filtrate to give a solution containing 50 to 300 µl of total amino acids.
5. Transfered 1 ml of this solution to a 25 ml volumetric flask. Added 10 ml of 50 % (v/v) glycerol, 2 ml of PH 6.0 M phosphate buffer and 1 ml of ninhydrin reagent. Also prepared a reagent blank and lysine HCl (50 to 300 µl).
6. Heated in a boiling water bath for 30 minutes and transfered to a cold-water bath and then cooled to room temperature
7. Made to volume with distilled water and after 15 minutes read absorbance using UV Spectrophotometer at 570 nm, set the instrument to zero absorbance with the regent blank. Corrected the absorbance of sample by subtracting absorbance of sample blank.

The lysine HCL concentration in the sample was estimated using the Lysine HCl absorbance value and the concentration and absorbance values of the lysine HCl standard obtained by using the formula

$$\text{Lysin HCl concentration} = \frac{\text{Lysine HCl absorbance} \times \text{Std. concentration}}{\text{Std. absorbance}}$$

g) Estimation of Fatty acid content (Cox & Pearson, 1962)

The free fatty acid was estimated by titrating it against KOH in the presence of phenolphthalein indicator. The acid number is defined as the mg KOH required to neutralizing the free fatty acids present in 1gm of sample. The free fatty acids content is expressed as oleic acid equivalents.

Materials Required

1% phenolphthalein in 95% ethanol

N potassium hydroxide.

Neutral solvent: 25ml of ether, 25ml of 95% alcohol and 1ml of 1% phenolphthalein solution was mixed and neutralized with N/10 alkali.

Procedure: Fatty acid content was estimated by the method of Cox and Pearson (1962). Dissolved 1 to 10g of sample in 50ml of the neutral solvent in a 250 ml conical flask. Added few drops of 1% phenolphthalein. Titrated the content against 0.1N potassium hydroxide. Shaked constantly until a pink color, which persists for fifteen seconds, was obtained.

Calculation

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titer value x Normality of KOH x 56.1}}{\text{Weight of the sample (g)}}$$

Fatty acid was calculated as oleic acid using the equation 1ml N/10KOH =0.028g oleic acid.

h) Estimation of Total Dietary Fibre Content (Prosky *et al*., 1988)

The sample was defated with petroleum ether. Recorded the loss of weight due to fat removal and made appropriate correction to the final % dietary fiber. Used distilled or deionized water to prepare reagent solutions. With each assay, run blanks along with samples to measure any contribution from reagents to residue.

Weighed out triplicate 1.0g samples accurately into 400 ml tall-form beakers.

1. Added 40 ml MES-TRIS blend buffer solution (pH 8.2) to each beaker.
2. Stired on magnetic stirrer until sample completely dispersed in solution.

3. Incubated with heat-stable α-amylase: Added 50 µl heat-stable α-amylase solution while stirring at low speed.
4. Covered each beaker with aluminium foil and incubated for 30 min with continuous agitation in a shaking water bath at 98-100°C.
5. Removed all sample beakers from shaking water bath and cooled to 60°C.
6. Scraped the ring around beaker and gels in bottom of beaker with spatula and rinsed side wall of beaker and spatula with 10 ml distilled water by using pipette.
7. Adjusted temperature of water bath to 60°C by draining some of hot water from water bath and adding cold water.
8. Added 100 µl protease solutions to each sample and covered with aluminium foil and incubated for 30 min with continuous agitation in a shaking water bath at 60±1°C.
9. Removed sample beakers from shaking water bath. Dispensed 5 ml of 0.56 1N HCl solution into sample while stirring.
10. pH adjusted to 4.1-4.8 by adding either 5% NaOH solution or 5% HCl solution.
11. Added 200 µl amyloglucosidase solutions while stirring on magnetic stirrer.
12. Covered with aluminium foil and incubated for 30 min with continuous agitation in a shaking water bath at 60±1°C.
13. To each sample, added 225 ml 95% EtOH pre-heated to 60°C. Ratio of EtOH volume to sample volume should be 4:1.
14. Covered all samples with aluminium foil and incubatedd at room temperature for 60 min to allow precipitate to form.
15. Tared the crucible containing Celite to nearest 0.1 mg.
16. Wet and redistributed the bed of Celite in the crucible using 15 ml of 78% EtOH from wash bottle.
17. Applied suction to crucible to draw Celite onto fritted glass as an even mat.
18. Quantitatively transfered all the precipitate and suspension from each beaker by using a wash bottle with 78% EtOH and a rubber spatula, to its respective crucible.
19. Used vacuum and washed the residue successively with two 15 ml portions of (a) 78% EtOH, (b) 95% EtOH and (c) Acetone.

20. Dried the crucible containing residue overnight in an air oven at 103°C.
21. Cooled the crucible in desiccators for approximately 1 h.
22. Weighed crucible containing dietary fibre residue and Celite to nearest 0.1 mg.

To obtain residue weight, subtracted tared weight, i.e., weight of dried crucible from Celite.

Protein and ash determination: One residue sample and the corresponding blank were analyzed for protein using Kjeldahl method. Used N x 6.25 as conversion factor for all cases to calculate gram of protein. For ash analysis, incinerated the second residue sample and corresponding blank for 5 hours at 525°C. Cooled in desiccators and weighed to nearest 0.1 mg. Subtracted crucible and Celite weight to determine ash content.

Calculations:

$$\text{Total Dietary Fibre (\%)} = \frac{\frac{R1 + R2}{2} - p - A - B}{\frac{m1 + m2}{2}} \times 100$$

Where, R1 - residue weight 1 from m1; R2 - residue weight 2 from m2; m1 - sample weight 1; m2 - sample weight 2; p - protein weight from R_1; A - ash weight from R_2 and B - blank

$$B = \frac{BR_1 + BR_2}{2} - BP - BA$$

Where, BR- blank residue; BP - blank protein from BR_1 and BA - blank ash from BR_2.

Statistical Analysis

The mean value and standard deviation of various parameters in the study were determined and compared statistically by using Duncan analysis of SPSSv16.0

RESULTS AND DISCUSSION

Vitamins A, K, B6, B3 and B9

The impact of Cr^{VI} stress on the content of vitamins A, K, B6, B3 and B9 in *Amaranthus dubius* plant is depicted in the table 7.1**.** The initial levels of Cr^{VI} stress up to a concentration of 20mg/kg soil caused an increase in Vit.A content over the control and then onwards started to decline. The highest Vit.A content of 0.82µg/g tissue recorded at 20mg Cr^{VI} treatment is recording 31.71% increase and the lowest content of 0.31µg/g obtained at 70mg Cr^{VI} treatment is recording a decrease of 44.64% over the control treatment. The enhancement as well as the decline in the content of vitamin A is progressive in nature (figure 7.1). Whereas the content of vitamins K, B6 and B3 recorded only progressive decline from the lowest Cr^{VI} stress level to the highest, with certain exceptions with respect to Vit.B6 and Vit.B9 content (figure 7.2, 7.3, 7.4 & 7.5). The highest content of 8.37µg, 1.37µg, 4.33 µg and 0.30µg recorded at lowest stress of 1mg Cr^{VI} treatment respectively for vitamin K, B6, B3 and B9 are 0.71%, 0%, 0.92% and 0% decrease respectively over the control. Similarly, the lowest content of 4.80µg, 0.37µg, 2.20µg and 0.14µg respectively for vitamin K, B6, B3 and B9 obtained at 70mg Cr^{VI} treatment and recorded a decrease of 43.06%, 72.99%, 49.66% and 53.33% respectively over the control treatment**.** The reduction of Vit.A recorded at the highest Cr^{VI} stress of 70mg Cr^{VI} treatment differ significantly lower over the content of control and other Cr^{VI} stress treatments whereas the highest increase recorded at 20mg Cr^{VI} treatment is insignificant over 10mg Cr^{VI} treatment while differences over others are significant (table 7.2). The highest reduction obtained for Vitamins K, B6, B3 and B9 at 70mg Cr^{VI} stress do not differ significantly over the content obtained at 50mg Cr^{VI} stress. Similarly, the differences obtained among the contents of vitamins K, B3 and B9 at 1mg and 5mg Cr^{VI} stress and their difference over control differ only insignificantly while with respect to Vit.B6 in addition to the above Cr^{VI} stresses, 10mg Cr^{VI} stress also caused no significant difference (table 7.3, 7.4, 7.5 & 7.6).

Table 7.1: The impact of Cr^{VI} stress on the content of various vitamins in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Vitamin content in µg/g									
	Vit A	*SD*±	Vit K	*SD*±	Vit B6	*SD*±	Vit B3	*SD*±	Vit B9	*SD*±
0mg (Control)	0.56	*0.06*	8.43	*0.06*	1.37	*0.06*	4.37	*0.06*	0.30	*0.02*
1mg	0.57	*0.06*	8.37	*0.06*	1.37	*0.06*	4.33	*0.12*	0.30	*0.01*
5mg	0.69	*0.04*	8.20	*0.10*	1.30	*0.10*	4.30	*0.10*	0.30	*0.01*
10mg	0.73	*0.05*	8.03	*0.06*	1.20	*0.10*	4.03	*0.21*	0.26	*0.02*
20mg	0.82	*0.07*	7.70	*0.30*	1.03	*0.06*	3.67	*0.21*	0.26	*0.01*
30mg	0.61	*0.10*	7.33	*0.15*	0.73	*0.15*	2.83	*0.25*	0.18	*0.04*
50mg	0.51	*0.06*	5.00	*0.40*	0.50	*0.10*	2.40	*0.10*	0.14	*0.02*
70mg	0.31	*0.09*	4.80	*0.20*	0.37	*0.06*	2.20	*0.10*	0.14	*0.02*

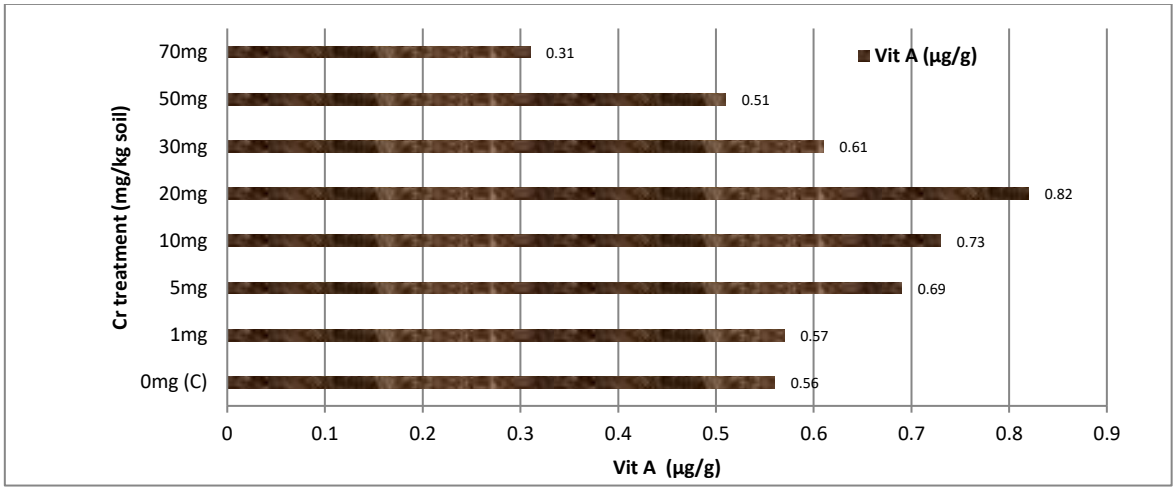

Figure 7.1: Vit A content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 7.2: Duncan analysis of Vitamin A content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	.31				
7 (50mg)		.51			
1 (Control)		.56			
2 (1mg)		.57	.57		
6 (30mg)		.61	.61	.61	
3 (5mg)			.69	.69	
4 (10mg)				.73	.73
5 (20mg)					.82
Sig.	1.000	.103	.055	.061	.099

Means for groups in homogeneous subsets are displayed.

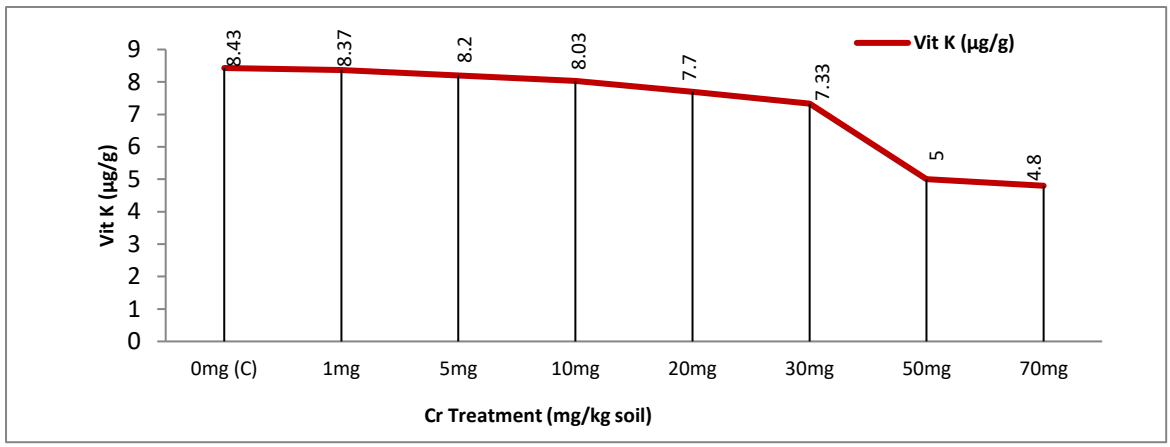

Figure 7.2: Vit K content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 4.3: Duncan analysis of Vitamin K content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	4.80				
7 (50mg)	5.00				
6 (30mg)		7.33			
5 (20mg)			7.70		
4 (10mg)			8.03	8.03	
3 (5mg)				8.20	8.20
2 (1mg)				8.37	8.37
1 (Control)					8.43
Sig.	.248	1.000	.063	.075	.203

Means for groups in homogeneous subsets are displayed

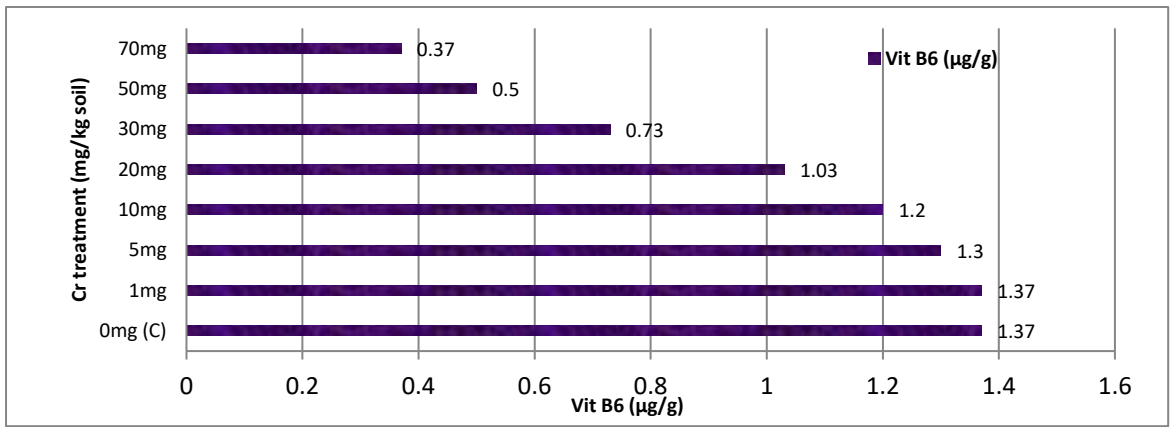

Figure 7.3: Vit B6 content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 7.4: Duncan analysis of Vitamin B_6 content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	.37			
7 (50mg)	.50			
6 (30mg)		.73		
5 (20mg)			1.03	
4 (10mg)				1.20
3 (5mg)				1.30
1 (Control)				1.37
2 (1mg)				1.37
Sig.	.093	1.000	1.000	.055

Means for groups in homogeneous subsets are displayed

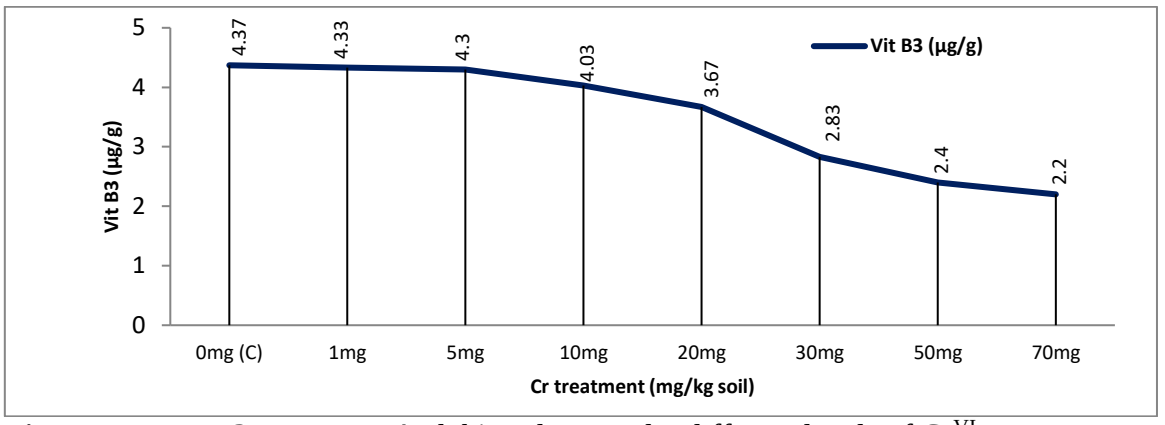

Figure 7.4: Vit B3 content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 7.5: Duncan analysis of Vitamin B_3 content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	2.20				
7 (50mg)	2.40				
6 (30mg)		2.83			
5 (20mg)			3.67		
4 (10mg)				4.03	
3 (5mg)				4.30	4.30
2 (1mg)					4.33
1 (Control)					4.37
Sig.	.138	1.000	1.000	.054	.629

Means for groups in homogeneous subsets are displayed.

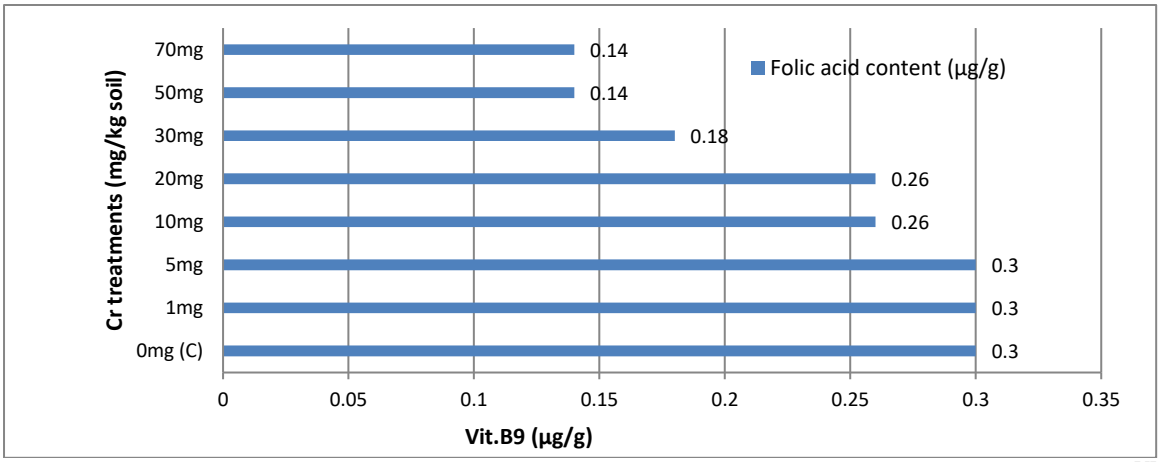

Figure 7.5: Folic acid (Vit. B9) content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 7.6: Duncan analysis of Folic acid (Vit. B9) content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05				
	1	2	3	4	5
8 (70mg)	.14				
7 (50mg)	.14				
6 (30mg)		.18			
5 (20mg)			.26		
4 (10mg)			.26	.26	
2 (1mg)				.30	.30
3 (5mg)				.30	.30
1 (Control)					.30
Sig.	.668	1.000	.830	.054	.839

Means for groups in homogeneous subsets are displayed

The antioxidant property of vitamins like Vit.C and Vit.E and their role in human health is well explored and established, whereas other vitamins such as Vit.A, Vit.K, Vit.B6, Vit.B3 etc. have been little attended and appraised for their role as an antioxidant in connection with health. This may be due to short of information regarding their direct involvement as antioxidant. However, few indirect roles such as constituent of coenzyme and coenzyme of redox enzymes for stimulating the antioxidant activity have been reported. Vit. A is reported to have some involvement in the regulation of antioxidant genes for inducing and enhancing the activities of superoxide dismutase and glutathione transferase (Gad *et al*., 2018; Malivindi *et al*., 2018). The vitamin K produced by leafy plants called Phylloquinone (vitamin K1) is essential for plant photosynthesis. Further, it

is reported to have the capacity to reduce the intensity of injury that can occur due to the depletion of glutathione by the increased generation of reactive oxygen species under stress conditions (Li *et al*., 2003). Vitamin B6 (Pyridoxine) is a water-soluble vitamin, which is important for carbohydrate, fat and protein metabolism and this vitamin is reported to have scavenging activity of free radicals like hydroxyl, superoxide and oxygen (Higashi-Okai, 2006). Anand (2005) reported the antioxidant activity of Vitamin B6 in response to chromium induced oxidative stress, as it stimulates the production of antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase etc. Vitamin B3 is another important water-soluble vitamin which is also known as Niacin. NAD and NADP are the major forms of vitamin B3 which are involved in the generation of energy from proteins and carbohydrates (Leskova *et al*., 2006). NAD, NADP, and niacin-containing enzymes are reported to have the capacity to protect or reduce the damage to the cells which are under oxidative stress conditions (Lanska, 2010). The water-soluble Vitamin B9 is reported to have protective role against lipid peroxidation and their effects have a comparable antioxidant property with vitamin C and vitamin E (Gliszczynska, 2007).

The increase of Vit.A content in *A. dubius* plants which are subjected to comparatively lower level Cr^{VI} stress and the decline in the content at comparatively higher level Cr^{VI} stress and similarly, the progressive degradation in the content of Vit.K, B6, B3 and B9 along with the increasing level of Cr^{VI} stress indicate the influence of oxidative stress induced by the generation of reactive oxygen species under Cr^{VI} toxicity. The increased content of Vit.A at lower level Cr^{VI} stress might be due to some stimulatory action on the enzymes or genes involved in their biosynthetic pathways, whereas the reduction in the content of various vitamins at higher level Cr^{VI} stress can be attributed to their utilization in the scavenging activity of free radicals to reduce the intensity of oxidative damage.

Lysine content

The impact of different levels of Cr^{VI} stress on lysine content in *Amaranthus dubius* is depicted in the table 7.7. The lysine content recorded a progressive reduction along with the increase in level of Cr^{VI} stress from 1mg Cr^{VI}/kg soil treatment to 70mg Cr^{VI}/kg soil (figure 7.6). The differences in reduction due to various concentrations of Cr^{V1} with

respect to control are found insignificant up to 20mg stress. The highest reduction of 69.92% over the control is recorded at 70mg Cr^{VI} stress which showed no significant difference with 50mg Cr^{VI} stress, while differences over the rest are significant (table 7.8).

Table 7.7: The effect of Cr^{VI} stress on the Lysine content in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Lysine (mg/g)	SD±
0mg (Control)	1.23	0.06
1mg	1.17	0.06
5mg	1.17	0.15
10mg	1.13	0.06
20mg	1.00	0.20
30mg	0.80	0.20
50mg	0.43	0.15
70mg	0.37	0.06

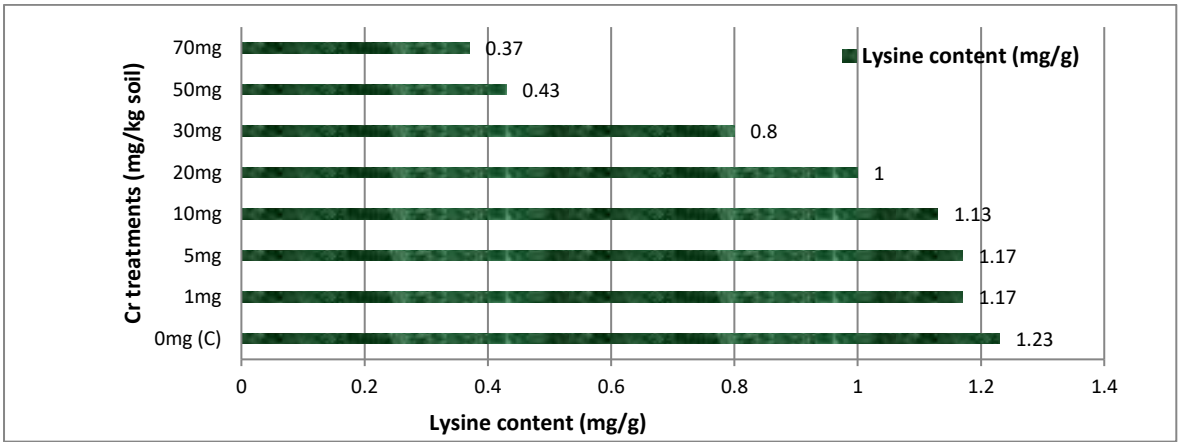

Figure 7.6: Lysine content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 7.8: Duncan analysis of lysine content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	.3667		
7 (50mg)	.4333		
6 (30mg)		.8000	
5 (20mg)		1.0000	1.0000
4 (10mg)			1.1333
2 (1mg)			1.1667
3 (5mg)			1.1667
1 (Control)			1.2333
Sig.	.546	.083	.067

Means for groups in homogeneous subsets are displayed.

There are reports that, when metal Cr interacts with enzymatic proteins, it gets binded on to specific functional groups and may cause deactivation of enzymes, which leads to disruption in the enzymatic activity of proteins (Gupta *et al*., 2009; Gupta *et al*., 2010). The present study observations on the lysine content of *A. dubius* plants are in agreement with this report. The reduction in the lysine content in *A. dubius* plants subjected to Cr^{VI} stresses might be due to the interference of Cr^{VI} in the biosynthetic pathway of lysine by altering the activities of key enzymes such as diaminopimelate epimerase, dihydrodipicolinic acid synthase, diaminopimelate decarboxylase etc. which are important to carry out the overall synthesis of lysine (Chatterjee *et al*., 1994).

Fatty acid content

Effect of different concentrations of hexavalent chromium on the fatty acid content of *Amaranthus dubius* is shown in table 7.9. Fatty acid content showed a decreasing trend with progressive increase in Cr^{VI} concentration. Reduction in fatty acid content is more prominent from 20 mg Cr^{VI} treatment per kg soil onwards. Maximum reduction is recorded at the highest concentration of 70mg Cr^{VI}/ kg soil while the least reduction is recorded at 5mg Cr^{VI}/ kg soil and which are 34.2% and 2.1% respectively; however, the treatment of 1mg Cr^{VI} did not contribute to any change in concentration of fatty acid content over control (figure 7.7). The differences in fatty acid content induced by lower level Cr^{VI} concentration of 1mg, 5mg and 10mg are not differing significantly among themselves and over control. Similarly, the reduction in fatty acid content induced by higher level Cr^{VI} concentration of 70mg, 50mg and 30mg did not differ significantly among themselves while their differences over control and rest of the treatments are significant (table 7.10).

Table 7.9: The effect of Cr^{VI} stress on the fatty acid content in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Total Fatty acid	SD±
0mg (Control)	0.187	0.01
1mg	0.187	0.02
5mg	0.183	0.02
10mg	0.180	0.02
20mg	0.157	0.01
30mg	0.140	0.02
50mg	0.130	0.02
70mg	0.123	0.02

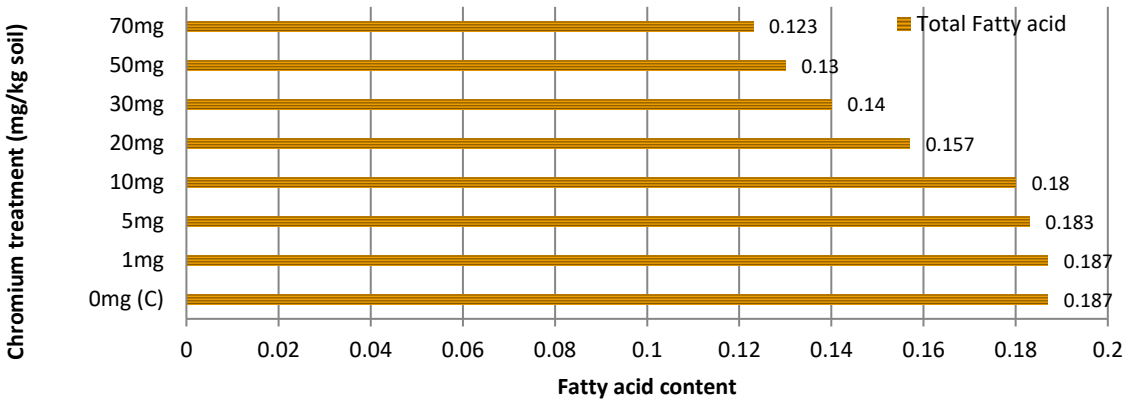

Figure 7.7: Fatty acid content in *A. dubius* plants under different levels of Cr^{VI} stress

Table 4.10: Duncan analysis of fatty acid content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05			
	1	2	3	4
8 (70mg)	.123			
7 (50mg)	.130	.130		
6 (30mg)	.140	.140		
5 (20mg)		.157	.157	
4 (10mg)			.180	.180
3 (5mg)			.183	.183
1 (Control)				.187
2 (1mg)				.187
Sig.	.235	.065	.065	.639

Means for groups in homogeneous subsets are displayed.

The decrease of fatty acid content in *A. dubius* plants under Cr^{VI} stresses observed in the study may be due to oxidative stress induced decomposition of polyunsaturated fatty acids. This may be considered as one of the potential parameters due to heavy metals toxicity. The present inferences are in similar lines with the observations of Guédard *et al*. (2008) in *Lactuca sativa* and Fikriye (2013) in *Phaseolus vulgaris* seedlings which when exposed to different concentrations of heavy metals, showed a decrease in fatty acid composition linearly with increased heavy metal concentration.

Total dietary fibre content (TDF)

The effect of different concentrations of Cr^{VI} on total dietary fibre content in *Amaranthus dubius* reveals a negative correlation exists between total dietary fibre content and

concentration of heavy metal Cr^{VI} (table 7.11) The investigation observed the highest TDF content among all treatments is obtained in the lowest concentration of 1mg Cr^{VI}/ kg soil, which is 1.52% decrease over control and the TDF content further decreases as the concentration of Cr^{VI} increases. The decrease is more prominent from treatment of 20mg Cr/ kg soil onwards and the maximum reduction of TDF content is recorded in plants treated with highest concentration of 70mg Cr^{VI}, which is about 40% decrease over control (figure 7.8). The reduction in the TDF content induced by lower level Cr^{VI} stresses of 1mg, 5mg and 10mg over control as well as the differences in TDF content among themselves are insignificant. However, the reduction caused by higher level Cr^{VI} stresses of 70mg, 50mg and 30mg concentrations are significantly higher over control and rest of the Cr^{VI} stresses while the differences among themselves are insignificant (table 7.12).

Table 7.11: The effect of Cr^{VI} stress on total dietary fibre content in *A. dubius* plant

Treatment Cr^{VI} / kg soil	Total Dietary fibre content	SD±
0mg (Control)	43.33	0.58
1mg	42.67	1.15
5mg	41.67	1.15
10mg	40.00	1.73
20mg	34.00	4.00
30mg	28.33	4.16
50mg	26.33	3.21
70mg	26.00	2.00

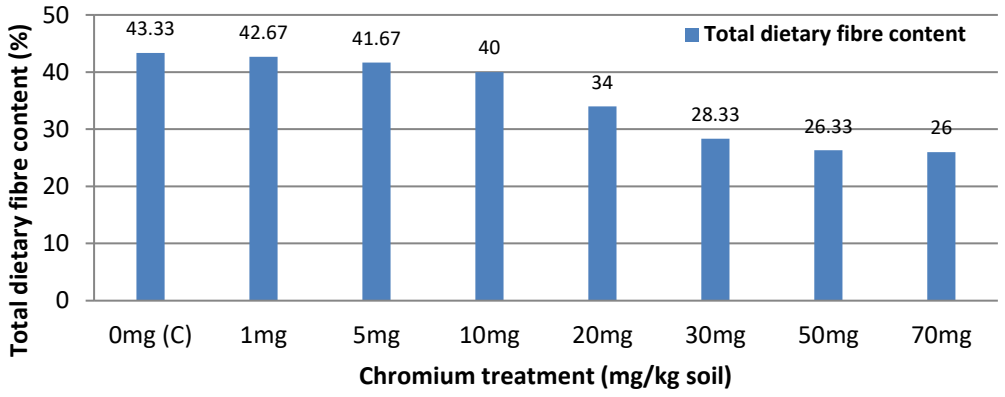

Figure 7.8: Impact of Cr^{VI} stress on the total dietary fibre content in *A. dubius* plants

Table 7.12: Duncan analysis of total dietary fibre content in *A. dubius* plants treated with Cr^{VI}

Treatment Code Cr^{VI} / kg soil	Subset for alpha = 0.05		
	1	2	3
8 (70mg)	26.0000		
7 (50mg)	26.3333		
6 (30mg)	28.3333		
5 (20mg)		34.0000	
4 (10mg)			40.0000
3 (5mg)			41.6667
2 (1mg)			42.6667
1 (Control)			43.3333
Sig.	.311	1.000	.165

Means for groups in homogeneous subsets are displayed.

The present study results are in conformity with earlier findings of Gill and Saggoo (2010) in turnip plants which are subjected to chromium stress. The Cr^{VI} toxicity induced reactive oxygen species which degrade important cellular components by inducing oxidative stress leading to low utilization of nitrogen and the reduction in photosynthesis might be the reason for stunted plant growth and reduction in TDF content.

CONCLUSION

The study reveals the impact of hexavalent chromium (Cr^{VI}) stress on the green leafy vegetable *Amaranthus dubius* by causing alterations in the content of selected nutritional components compared to control. The majority of the components such as Vit K, Vit B3, amino acid lysine and dietary fiber generally exhibited a decreasing trend in their content with increasing Cr^{VI} stress from the lowest stress level of 1mg Cr^{VI}/kg soil to the highest of 70mg Cr^{VI}/kg soil and this clearly indicates a negative correlation. The exceptions include Vit A content, which showed an increasing trend up to 20mg Cr^{VI}/kg soil and then starts to decrease, while the content of Vit B9 remains unchanged at 1mg & 5mg Cr^{VI} stress whereas Vit B6 and fatty acid content remain uninfluenced only at 1mg Cr^{VI}/kg soil and then the content of all these components exhibited a decreasing trend with further increasing Cr^{VI} stress. The alterations in the nutritional values of selected components in the study indicate clear influence of heavy metal Cr^{VI} on the nutritional quality of the vegetable *A. dubius*. The drastic and significant decrease in the content of most of the selected parameters inferred in the study, particularly at higher level stress of 50mg & 70mg Cr^{VI} revealed Cr^{VI} toxicity induced oxidative damage and reduction in overall nutritional value of the crop. Vit A, Vit B3 and fatty acids which are reported to have some antioxidant role directly or indirectly in plants against stresses and their concentration changes recorded in *A. dubius* in the study might be due to their utilization in the radical scavenging activity to reduce oxidative damage. The study further suggests that the alterations in the concentration of nutritional components inferred in the study can be used as a bioindicator of hexavalent chromium (Cr^{VI}) stress in *A. dubius* in the future.

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CHAPTER-8

ANALYSIS OF DIFFERENTIAL EXPRESSION OF GENES (DEGs) UNDER HEXAVALENT CHROMIUM (Cr^{VI}) STRESS

INTRODUCTION

The plant cells are generally perceived different types of stress stimuli through changes like variation in turgor pressure or membrane receptor activity and such extracellular signals are then transformed into intracellular signals through the formation of second messengers that can stimulate or can suppress the transcription factors (TFs) or protein kinases (PKs), results in differential expression of specific genes (Huang *et al*., 2012). Researchers are now worldwide using the transcriptome sequencing and RT qPCR analysis to identify and quantify differentially expressed genes (DEGs) under various stress conditions (Shi *et al*., 2017; Fowler & Thomashow, 2002). The oxidative stress due to accumulation of heavy metals can affects various biological growth processes leading to retarded growth, yield and quality of crop plants. Under metal stress conditions, the plants may have evolved several adaptive or tolerating mechanisms to reduce the impact of stress. The first line of defence is by inhibiting long distance transport by binding metal ions to the cell wall of roots or by hindering their uptake through apoplast pathway and further plants get started to adjust over time at cellular, organ, physiological, biochemical, and molecular levels, which are finally reflected as retarded morphological growth changes.

The increased level of reactive oxygen species production (ROS) under metal stress can cause lipid peroxidation involving higher level of destruction to the cell membrane, disturbances and damages to nucleic acids and chloroplast integrity leading to the disruption of cellular homeostasis (Karuppanapandian & Kim, 2013). This may even change the protein expression profiles affecting the normal physiology and metabolism which leading to aggravated cell death (Foy *et al*., 1978; Ouelhadj *et al*., 2006). The transcriptome profiling and RT qPCR analysis is the most scientific and genuine approach in the present scenario to get a better understanding of metal stress in plants

since it can reveal the expression of numerous genes and metabolic pathways involved (Martin *et al*., 2013). In order to get a better insight into the gene expression patterns of heavy metal stressed plants, the most reliable molecular approach is to analyse the differential gene expressions (DGEs) followed by Gene Ontology (GO) analysis. A comparative RNA sequence analysis of the control and metal stressed plants and consequent validation of the selected candidate genes can reveals a better understanding of the stress responsive mechanism involved at the molecular level. Although various studies have been conducted on chromium metal stress, the molecular mechanisms underlying the response of *Amaranthus dubius* when treated with toxic amount of chromium is not yet investigated. This study is carried out in this context to understand the molecular level changes in *Amaranthus dubius* in response to disruptions and alterations in the biological growth characteristics under hexavalent chromium (Cr^{VI}) stress by focussing on the following aspects

a) RNA sequence analysis of stressed and control plants to reveal Cr^{VI} toxicity induced differential gene expression patterns
b) Categorisation of differentially expressed genes (DEGs) as up-regulated and down-regulated based on their biological, cellular and molecular functions
c) Quantification of selected stress responsive candidate genes of interests using RTqPCR analysis

REVIEW OF LITERATURE

The growth, development and survival of plants in the natural environment are often affected by several adverse factors which are generally and collectively termed as ‘stresses. Abiotic stress and biotic stress are the two main categories of stress encountered by plants. Abiotic stress is basically due to physical or chemical circumstances like drought, heavy metal stress, high salt content, chilling injury etc. As a result of the advancement in the molecular biology technology, the study of molecular mechanism in plants with regard to unfavourable stress has emerged as an area of academic interest (Xiaojuan *et al*., 2020). At a specific stage of evolution of an individual organism, the level of expression of all genes in a particular cell, tissue, or organ is reflected as the expression of the transcriptome which in general is dynamic in nature. Under conditions of stress, the expression of the whole genome level from the whole transcriptional level can be disclosed via transcriptomics. This can contribute to the understanding of complex regulatory network linked with the flexibility and endurance of plants to stress (Xiaojuan *et al*., 2020).

Nowadays RNA sequencing emerges as a quantitative transcriptome profiling system (Mutz, 2013) and this can provide the transcriptome analysis of large sequence even without the fully sequenced reference genome (Gao, 2013). A comparative transcriptome analysis of *Nicotiana rustica* and *Nicotiana tobacum* revealed 173 and 710 differentially expressed genes (DEGs) in the leaves and roots of *N. rustica* while 576 and 1543 DEGs are obtained in the leaves and roots of *N. tobacum* when treated with $CdCl_2$ (Zhang *et al*., 2021). Next generation sequencing of transcriptome analysis of radish in response to chromium stress reveals differential expression of 2985 genes of which 1424 are upregulated and 1561 downregulated which are involved in metabolic process in response to abiotic stimulus (Xie *et al*., 2015).

The research in the changes of transcription profiling in *Arabidopsis thaliana* under metal Cr stress conducted by Klees *et al*. (2021) identified 238 upregulated genes and 858 downregulated genes in response to treatments, which indicates that the metal chromium has some serious effect the normal growth and development of Arabidopsis. In the study of Liu *et al*. (2017) in *Sedium plumbizincicola* using Cd identified a $SpHMA_3$ gene

responsible for heavy metal transport and detoxification, which helps in the normal growth of young leaves in the plant. In the studies conducted in *Raphanus sativus* L., transcription factors like WRKY33 are found significantly changed whereas the expression of ARF and ERF transcripts are seen decreased after treatment with Cd stress (Peng *et al*., 2021). The gene expression profiling conducted by Dubey *et al*. (2010) in rice under chromium stressed condition, ABC transporters, glutathione S transferase, cytP450, HSPs, Mate- efflux family proteins are found to be upregulated whereas majority of the genes of energy metabolism, phenyalanine metabolism, photosynthesis and cell cycle are downregulated as per gene ontology analysis performed. Comparative transcriptome profiling of two different *Brassica napus* cultivars under chromium toxicity revealed the increase in transcription factors related to antioxidant activity, hydrolase activity and also upregulated several proteins related to intracellular membrane bounded organelles (Gill *et al*., 2016). It has been found that under excess Cu, photosynthetic parameters like photosystem I, photosystem II, light harvesting chlorophyll binding proteins have reduced expression in Petunia plants.

The light-harvesting chlorophyll a/b-binding (LHCB) proteins are the apoprotein of the light-harvesting complex of photosystem II (PSII). LHCB proteins are normally associated with chlorophyll and xanthophyll and serve as the antenna complex which absorb sunlight and transfer the excitation energy to the core complexes of PSII to drive photosynthetic transport (Villegas *et al*., 1994). The researches on transcriptome analysis of *Phyllostachys edulis* annotated 35 genes as light harvesting chlorophyll ab binding protein and 9LHC like proteins in response to high light intensity (Zhao *et al*., 2016). Beta tubulin is a dimeric plant protein that contributes to the formation of microtubules, and major intracellular structures that are involved in the control of fundamental processes such as cell division, the polarity of growth, cell-wall deposition, intracellular trafficking and communication and the TUB genes which coding for Beta tubulin protein in plants are investigated extensively in connection with oxidative stress (Rao *et al*., 2016).

Superoxidase dismutase and catalase are two enzymes with very important roles in dealing with reactive oxygen species (ROS) that are probably formed during stress

conditions (Ara *et al*., 2013). Superoxide dismutase (SOD) is an enzyme that can convert O_2^- into hydrogen peroxide (H_2O_2). Three types of SOD with metal cofactors are known, Copper/Zinc (Cu/ZnSOD), Manganese (MnSOD), and Iron (FeSOD). MnSOD is found in eukaryotic mitochondria and peroxisomes, Cu/ZnSOD isoenzymes are found in the cytosol of plants, and FeSOD in the chloroplast (Montero *et al*., 2015). Catalases (CAT) are enzymes that can convert O_2^- into water and molecular oxygen. CAT gene encodes a small family of proteins including CAT1, CAT2, and CAT3 (Ara *et al*., 2013). H_2O_2 in smaller amounts involves in various signalling pathways, but in higher concentrations, it can get converted into ROS and damage DNA, cell membranes, and proteins. In a study, the chamomile plants were exposed to Chromium (III) for seven days, an increase in chromium accumulation was seen in roots along with a high concentration of ROS, thiols, and nitric oxide. While analysing the SOD and CAT activities at different concentrations, a decrease was observed whereas at higher chromium concentration, H_2O_2 level was irregularly varying and the concentration of SOD was high (Sharma *et al*., 2016). Glutathione S-transferase (GST) is an enzyme well known for their detoxification abilities, it protects biomolecules from the attack of reactive species (Evans *et al*., 2017, Srivastava *et al*., 2019). High throughput RNA sequencing and gene expression profile analysis related to Cd stress tolerance in Kenaf expressed an enhanced antioxidant enzyme activity in lower concentration of 10 mgL^{-1} and further a gradual decline in expression at 30 mgL^{-1} Cd stress (Chen *et al*., 2020). A transcriptome study in *Oryza sativum* treated with heavy metal chromium when compared to control showed differential expression of genes related to glutathione metabolism, transportation, and signalling (Srivastava *et al*., 2021). The RTqPCR analysis in *Withania somnifera* exposed to copper with varying concentrations reveals a progressive upregulation of SOD and CAT at 25µM and 50 µM whereas it shows a gradual decrease in fold of induction at further higher concentrations when compared to control. While at the same time, no significant amplification of gene expression is noticed with respect to POX in cells subjected to copper stress (Rout & Sahoo, 2013).

MATERIALS AND METHODS

Extraction of RNA

Total RNA Extraction Kit was designed for rapid purification of RNA from control and 70 mg chromium treated *Amaranthus dubius* plant samples using Trizol reagent (Thermo Fisher, USA). Liquid nitrogen was utilised for grinding 5 gm of plant tissue. Added 1ml of Trizol reagent and then transfered the mixture to a 2.0ml collection tube. Incubated for 5 minutes at room temperature (15-25˚C). Added 200µl of chloroform and inverted the mix for 15 seconds, and allowed the tube to stand for 10 minutes at room temperature (15-25˚C). After centrifugation, collected the upper aqueous phase containing RNA and then treated with 500µl of ice-cold isopropyl alcohol. Allowed the sample to stand for 10 minutes at room temperature (15-25˚C). The RNA precipitated out was washed with 75% of ethanol. After air drying, the RNA resuspended in 50µl of RNase-free water. Stored the RNA at lower temperature (-80˚C).

RNA Quantitation

To determine the RNA concentration and integrity, Qubit™ RNA XR Assay kit and Qubit™ RNA IQ Assay kit was used and estimated using Qubit®.4.0 Fluorometer.

Library preparation and purification of cDNA

1 µg total RNA with RIN value above 7 was used for library preparation using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® according to the manufacturer's protocol. For RNA-seq, the sequencing was done by enriching mRNA (using oligo dT beads) from the total RNA extracted and converted them to cDNA using random hexamer primers. The cDNA was then used for size selection, adaptor ligation and amplification during library preparation.

Transcriptome analysis

Transcriptome analysis on the impact of hexavalent chromium (Cr^{IV}) on *Amaranthus dubius* was done using plant samples from control and highest stress treatment of 70mg Cr^{IV}. The quality of sequences obtained for each sample was determined using FASTQC program. According to the quality report from FASTQC, the low-quality bases from the reads were removed. Average Q30 (Phred value) score was used as a cut-off to remove

low quality bases. Also, if any specific bias was observed in base composition, those bases were also trimmed. The low-quality bases were removed using Trimmomatic (version 0.39). Using the tool Cutadapt (version 2.1) the adapter sequences from the based trimmed reads were removed. After adapter trimming, Perl script was used to select the pre-processed reads having read length >=30 bases, as the reads less than 30 bases doesn't make any influence in alignment process. Then the reads were aligned against silva database using bowtie the aligner tool bowtie (version 2.4.2). The unaligned reads were considered for the rest of the analysis. The reads were aligned to reference genome *Amaranthus hypochondriacus* using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) version 2.1.0. More than 77% of the total pre-processed reads of both samples were mapped to the reference genome. The fasta and gene model files of reference genome is given in **table 8.1**.

Table 8.1: Reference Genome Information

Fasta File	*Amaranthus hypochondriacus*_459_v2.0.fa.gz
GFF File	*Amaranthus hypochondriacus*_459_v2.1.gene_exons.gff3.gz

The differential expression comparison between samples was done using "cuffdiff" program in the Cuffdiff tool. The up and down regulated genes with FPKM>=1 and p-value < 0.05 were identified from the "cuffdiff" result using custom made Perl script. The gene expression distribution plot, scatter plot and volcano plot were generated using Cummerbund R package. The *A. hypochondriacus* final annotation file was generated by combining the annotations from Phytozome and the GO annotation. All the differentially expressed genes were annotated based on the final annotation file. All the mapping and parsing were done using Perl scripts.

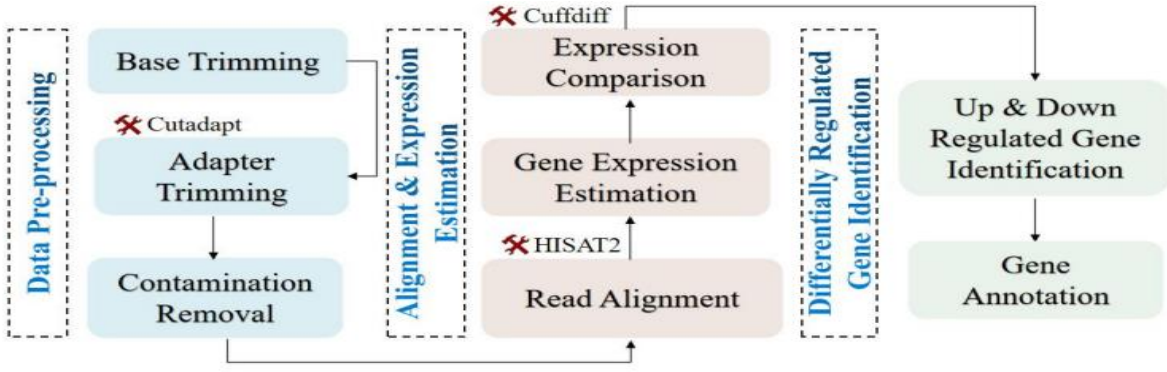


Figure 8.1: RNA-Seq analysis pipeline

Gene expression study using Real Time PCR analysis

cDNA synthesis

The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit was used for first strand cDNA synthesis for RT-PCR according to the manufacturer's protocol. Used 20ng of total RNA to generate first-strand cDNA as the initial step of a two-step RT-PCR protocol. Plant samples from control, plants treated with 1mg, 5mg, 10mg, 20mg, 30mg and 50mg Cr^{IV} stress were analysed in the real-time PCR.

Real Time PCR or qPCR analysis

RNA extraction was carried out and concentration of RNA was assessed by Qubit®.4.0 Fluorometer. 20ng of total RNA was used to synthesis cDNA, using Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit. Qiagen Rotor-Gene Q, along with QIAGEN kits were used for qPCR analysis. 1 μg total RNA with RIN value above 7 was used for library preparation using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® according to the manufacturer's protocol. Four genes of interest SOD, CAT, LHCB, GSTU21 were selected and analysed for their expression in comparison with the house-keeping gene β-tubulin and test samples of 1 mg, 5 mg, 10 mg, 20 mg, 30 mg, 50 mg Cr^{IV} treated plants and control. The Real-time PCR or qPCR, can provide a simple and elegant method for determining the amount of a target sequence or gene that is present in a sample. The threshold must be set in the amplification plot's linear phase. The Ct value increases as the amount of template decreases. Artifacts from the reaction mix or instrument, on the other hand, will cause template-independent changes in the Ct value. As a result, Ct values from PCR reactions run under different conditions or with different reagents cannot be directly compared. Amplification curve-based qPCR measurements are sensitive to background fluorescence.

The primers designed for PCR analysis is enlisted below:

GENES	SEQUENCES (5'-3')
SOD – F	5’ TTCTATTGTGGGGAGGGCTG 3’
SOD – R	5’ GACGAGCTTAACCTTGAAGACC 3’
CAT – F	5’ TCAAGCCGAACCCAAAATCC 3’
CAT – R	5’ ATTTGGGACGCCAATGGAAC 3’
GSTU21– F	5’ TCCATGCATCTTTTAGCCCA 3’
GSTU21– R	5’ TGGAGGGGATTCATTTGGGT 3’
Lhcb1*Ah1 – F	5’ GGCTGTGTTTTCCCGGAATT 3’
Lhcb1*Ah1 – R	5’-TCCACTGCACCCATCAAGAT- 3’
BTUB1 – F	5’ TTTTGGACAATCTGGGGCAG 3’
BTUB1 – R	5’ CTCCTCCCAATGAATGACAAACT 3’

The PCR program is:

Polymerase activation	95 °C	10 minutes	40 cycles
Denaturation	95 °C	15 sec	
Annealing/extension	55 °C	1 minute	

The CQ value, mean CQ, standard deviations, PCR efficiencies, and Fold of inductions are calculated for each gene with reference to the house-keeping BTUB gene.

RESULTS AND DISCUSSIONS

RNA extraction and quantification by Qubit

The total RNA extracted from treated samples and control of *Amaranthus dubius* are isolated using the Total RNA Extraction Kit for rapid purification of RNA using the Trizol reagent. The integrity of mRNA depends on rRNA quantity and quality. The RIN value shows the intactness of RNA which may range from one to ten during analysis. RIN 10 expresses the most intact RNA profile whereas RIN value 1 shows the most degraded RNA (Schroeder, 2006). In this study 1µg total RNA with RIN value above 7 is used for cDNA library preparation. The Qubit concentration obtained and RIN value of each sample are given in **table 8.2.**

Table 8.2: Qubit concentration and RIN value of samples

Sample	Concentration ng/µl	RIN Value
Control	68.8 ng/µl	8
1mg	18 ng/ µl	7.8
5mg	36.6 ng/ µl	8
10mg	36.2ng/ µl	8.2
20mg	6 ng/ µl	7.9
30mg	9.3 ng/ µl	8.2
50mg	3.6 ng/ µl	8.1
70 mg	56.4 ng/ µl	7.9

Transcriptome library preparation and purification of cDNA

The extracted RNA is fragmented and processed to prepare paired end cDNA libraries from both samples. Next generation sequencing library preparations are constructed using NEB Next® Ultra ^{TM} RNA Library Prep Kit for Illumina®. The mRNA enrichment using oligo dT beads as per mentioned in materials and methods and the quality of cDNA libraries synthesized are verified using bioanalyzer. Libraries prepared showed significant peak at 327 bp and 324 bp for control (C) and Test (T) (70mg Cr^{VI} treatment) respectively with a concentration of 10.7 and 6.16 ng/ µl with a total volume of 20 µl (**figure 8.2, 8.3 & 8.4**).

A1: Electronic Ladder

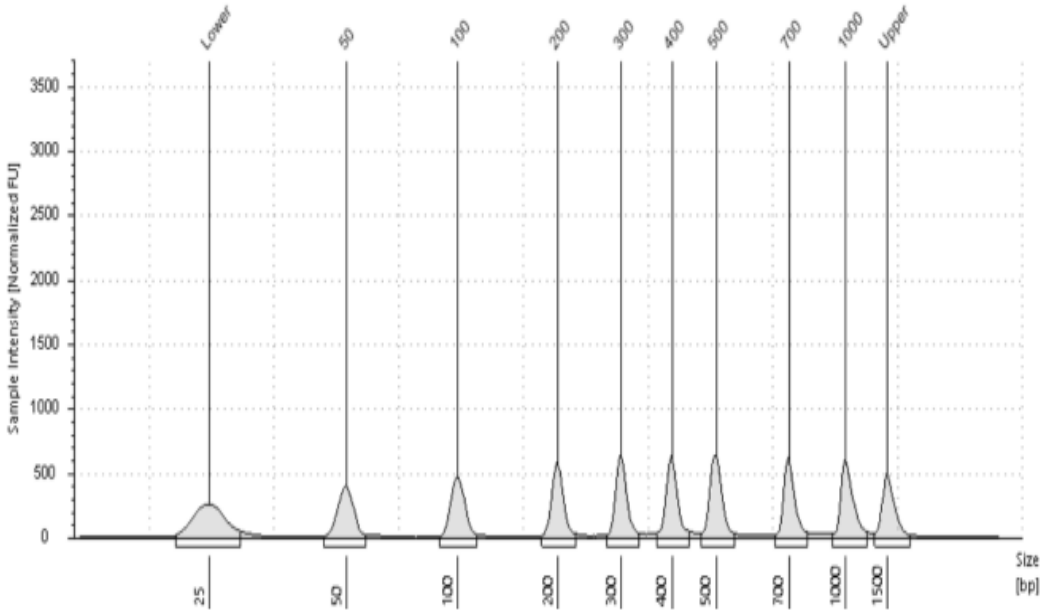


Figure 8.2: Standard ladder from TapeStation Profile

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	433	-	26700	-		Lower Marker
357	125	-	537	100.00		
1500	250	250	256	-		Upper Marker

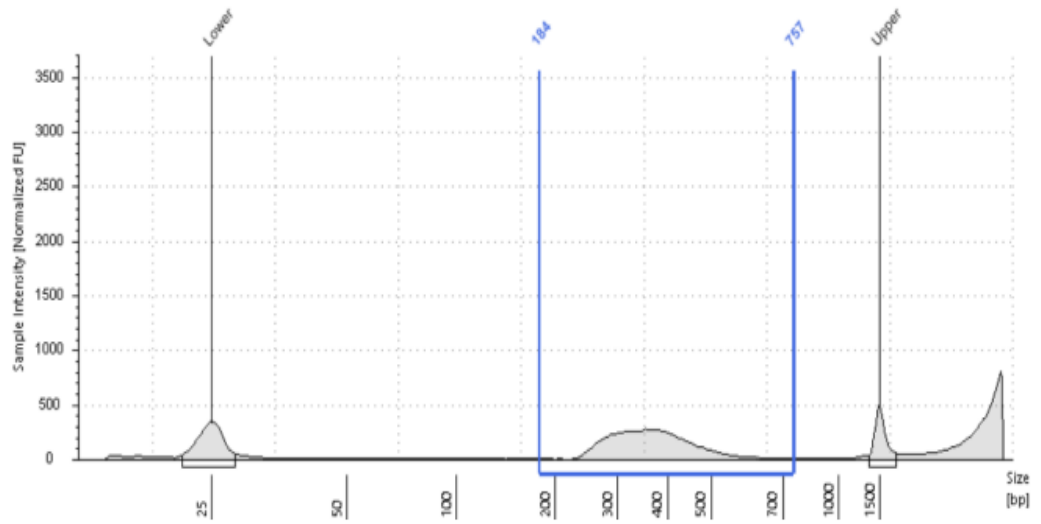


Figure 8.3: Control run in TapeStation to determine the size of cDNA library

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	440	-	27100	-		Lower Marker
324	844	-	4000	100.00		
1500	250	250	256	-		Upper Marker

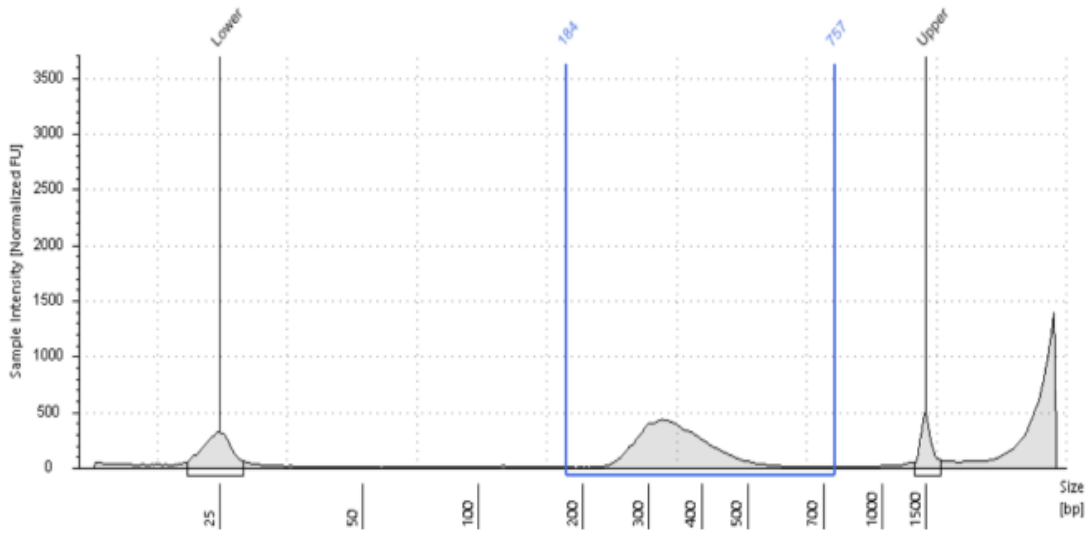


Figure 8.4: Test run (70mg) run in TapeStation to determine the size of cDNA library

Transcriptome analysis

The FASTQ quality information of the two samples are provided in **table 8.3**. The two samples, control (C) and 70mg treated *Amaranths dubius* are subjected to RNA extraction and RNA sequence analysis. Approximately 13.78Gb and 19.1GB data is obtained for the control and test samples respectively. High quality paired sequence reads of 45615610 for the control and 63245812 for the test is generated. The Q30 value of clean reads exceed 93.38% and 92.68% for both samples respectively, indicating high quality of sequencing result. All raw data from the libraries are deposited at the NCBI Sequence Read Archive (SRA) with the accession number **SUB11418745.**

Table 8.3: Raw read information

Sample Name :	C	T
Total Reads :	9,12,31,220	12,64,91,624
Total Paired Reads :	4,56,15,610	6,32,45,812
Raw Read Length :	151 x 2	151 x 2
Total Data :	13.78 (Gb)	19.1 (Gb)
Total A's :	3.91 (Gb) (28.39%)	5.38 (Gb) (28.18%)
Total C's :	2.99 (Gb) (21.76%)	4.18 (Gb) (21.87%)
Total G's :	3.08 (Gb) (22.34%)	4.4 (Gb) (23.03%)
Total T's:	3.78 (Gb) (27.44%)	5.13 (Gb) (26.87%)
Total Data >= Q30 :	93.38%	92.68%
Average GC% :	44.1%	44.89%

1. **Base composition and quality** - The average base composition and average base quality of Read1 (R1) and Read2 (R2) of the two samples and GC distribution plots are shown in figures 8.5 - 8.14**.** Read composition show a slight bias at start of the reads, which were removed during base trimming. The adapter sequences present in the reads were removed using cut adapt. After adapter trimming, reads less than 30bp were removed. The pre-processed reads then aligned to the Silva database in order to remove the rRNA contamination and to obtain only mRNA sequences for the further analysis.

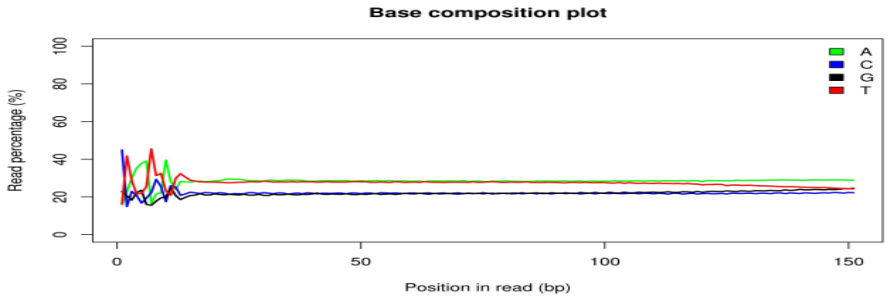


Figure 8.5: Average base composition of R1 reads of Control sample (C)

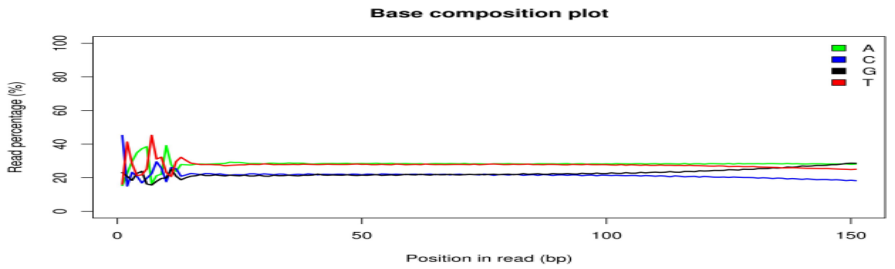


Figure 8.6: Average base composition of R2 reads of Control sample (C)

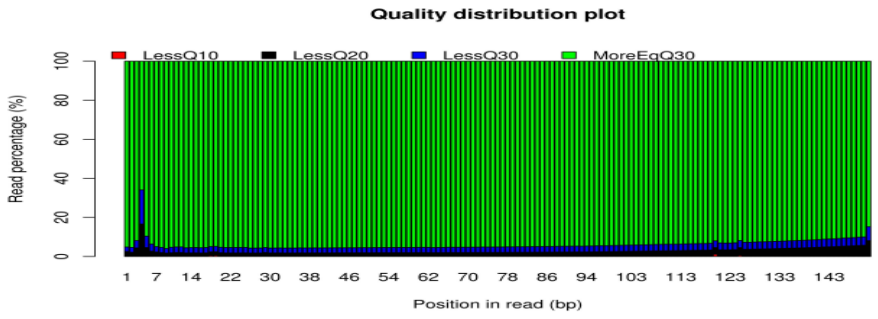


Figure 8.7: Average base quality of R1 reads of Control sample (C)

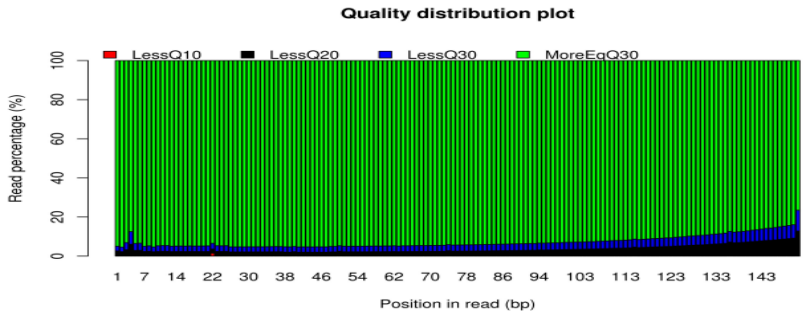


Figure 8.8: Average base quality of R2 reads of Control sample (C)

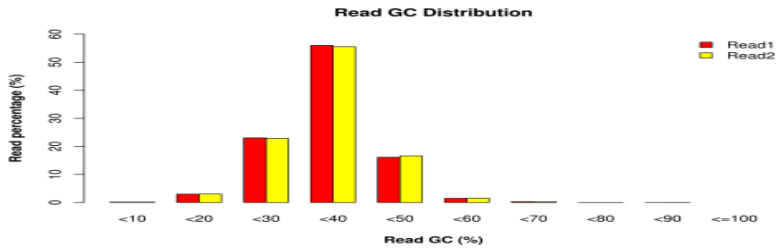


Figure 8.9: GC distribution of Control sample (C)

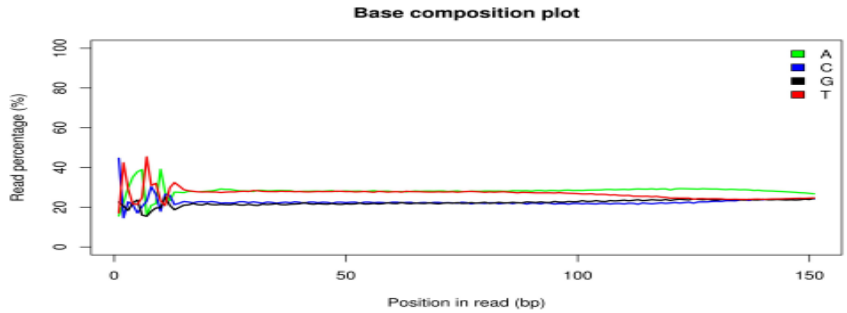


Figure 8.10: Average base composition of R1 reads of treated sample (T)

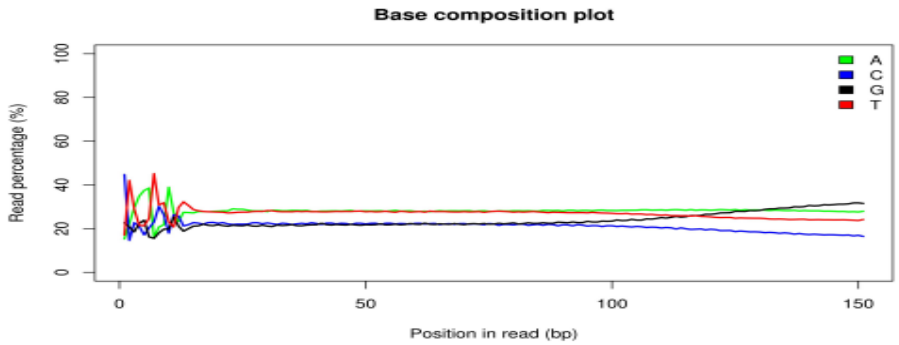


Figure 8.11: Average base composition of R2 reads of treated sample (T)

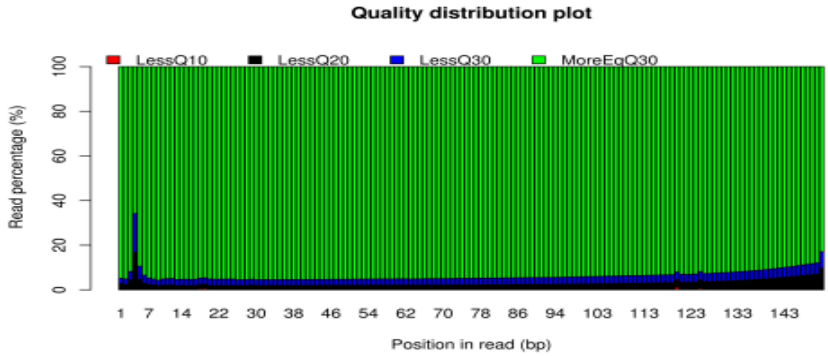


Figure 8.12: Average base quality of R1 reads of treated sample (T)

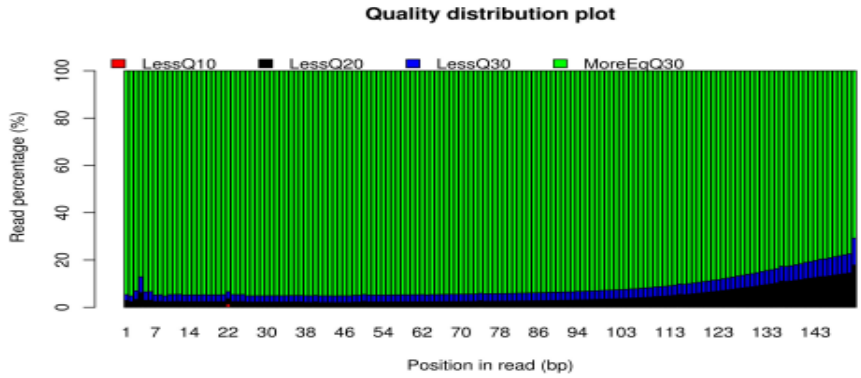


Figure 8.13: Average base quality of R2 reads of treated sample (T)

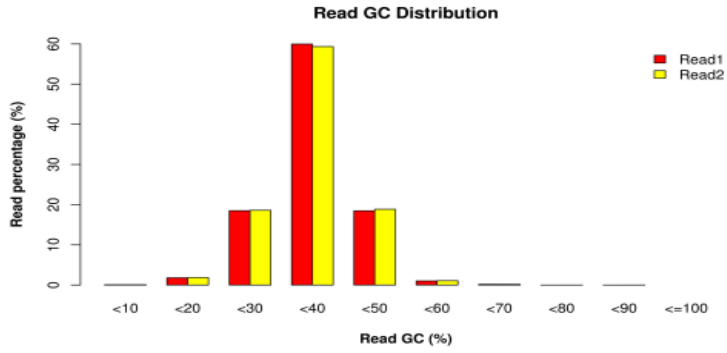


Figure 8.14: GC distribution of treated sample (T)

2. **Alignment to reference genome** –In the present investigation, the pre-processed reads of *Amaranths dubius* samples "C" and "T" are aligned to the reference *Amaranthus hypochondriacus* genome using RNA-Seq aligner HISAT2. In the case of control, 69930256 are mapped from a total of 90349170 reads and in case of test samples 97440080 are mapped from a total of 125276526 reads. More than 77% of the total pre-processed reads of both samples are mapped to the reference genome. The read alignment summary of all samples is shown in table 8.4.

Table 8.4: Read alignment summary

Sample Name :	C	T
Total reads :	9,12,31,220	12,64,91,624
Total pre-processed reads :	9,03,49,170	12,52,76,526
Total aligned reads :	6,99,30,256	9,74,40,080
Alignment% :	77.4%	77.78%

3. **Gene Expression Analysis** - The gene expression estimation was performed using cufflinks program. The estimated gene-expression distribution in FPKM (Fragment Per Kilo Per Million) unit is shown in figure 8.15. The correlation scatter plot between samples is shown in figure 8.16.

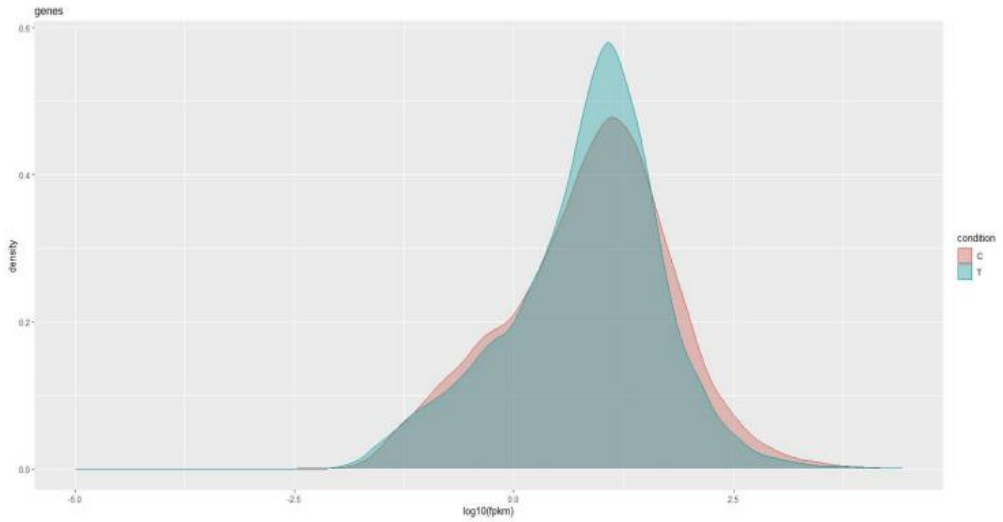


Figure 8.15: Gene expression distribution plot of the sample comparison

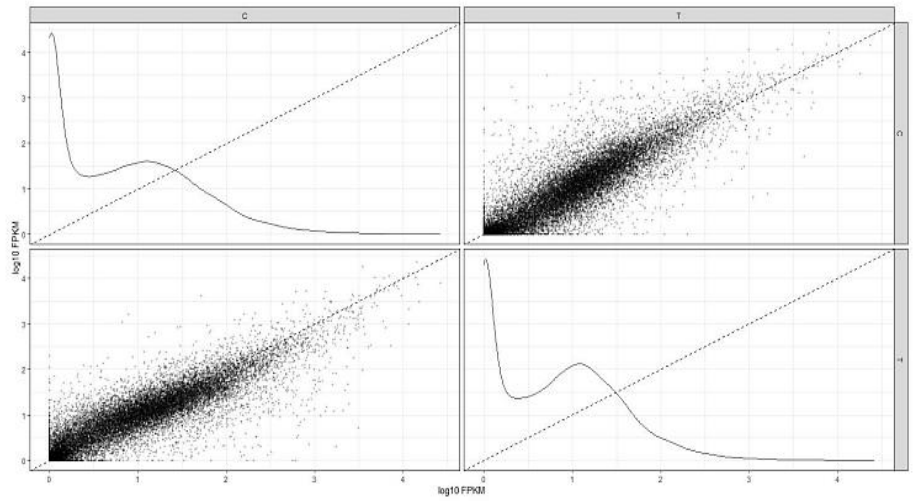


Figure 8.16: Sample correlation scatter plot of the sample comparison

4. **Identification of differentially expressed genes (**DEGs) **responsive to Cr Stress**

In order to identify the genes responding to heavy metal chromium stress treatment, the RNA sequence data are analysed for differential gene expressions, performed using cuffdiff program of cufflink package with default settings. DEGs determined at a threshold of log2 fold change ≥1 with p-value <= 0.05 of samples of selected genes is listed in appendix I. The sample comparison statistical analysis of Control vs Test generated 790 DEGs of which 307 are upregulated and 483 downregulated. The comparison statistics is given in table 8.5. The volcano plot of the sample comparison is shown in figure 8.17. Heat Map of genes with p-value cut off <= 0.05 is provided in figure 8.18.

Table 8.5: Sample comparison statistics

Comparison	Total differential expression genes	Up-regulated	Down-regulated
Control vs. Test	790	307	483

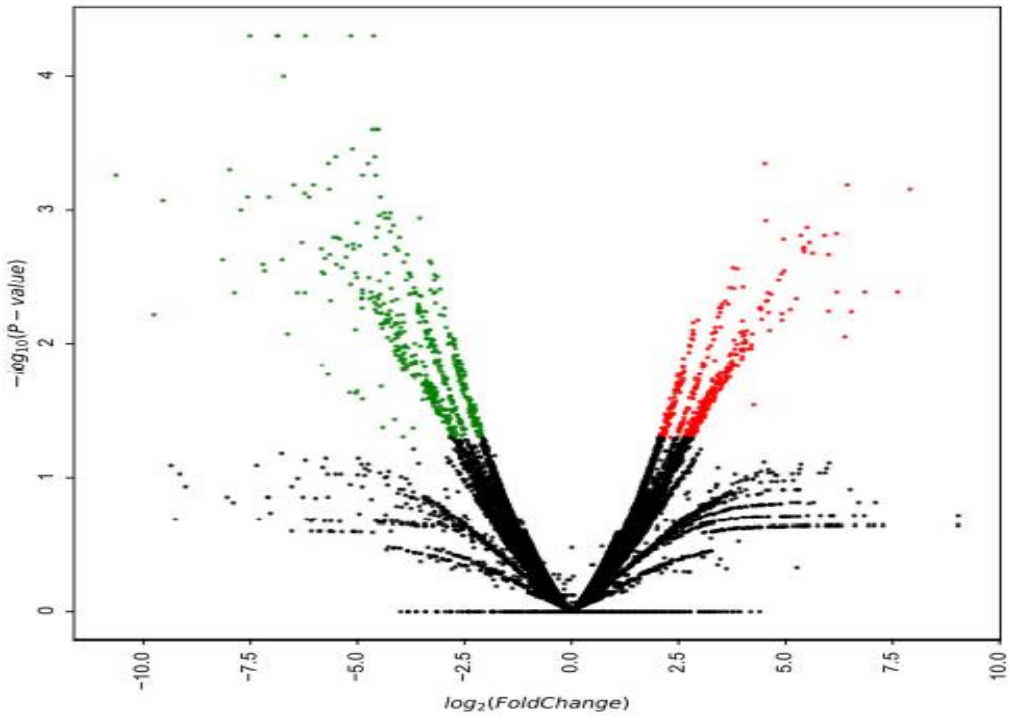
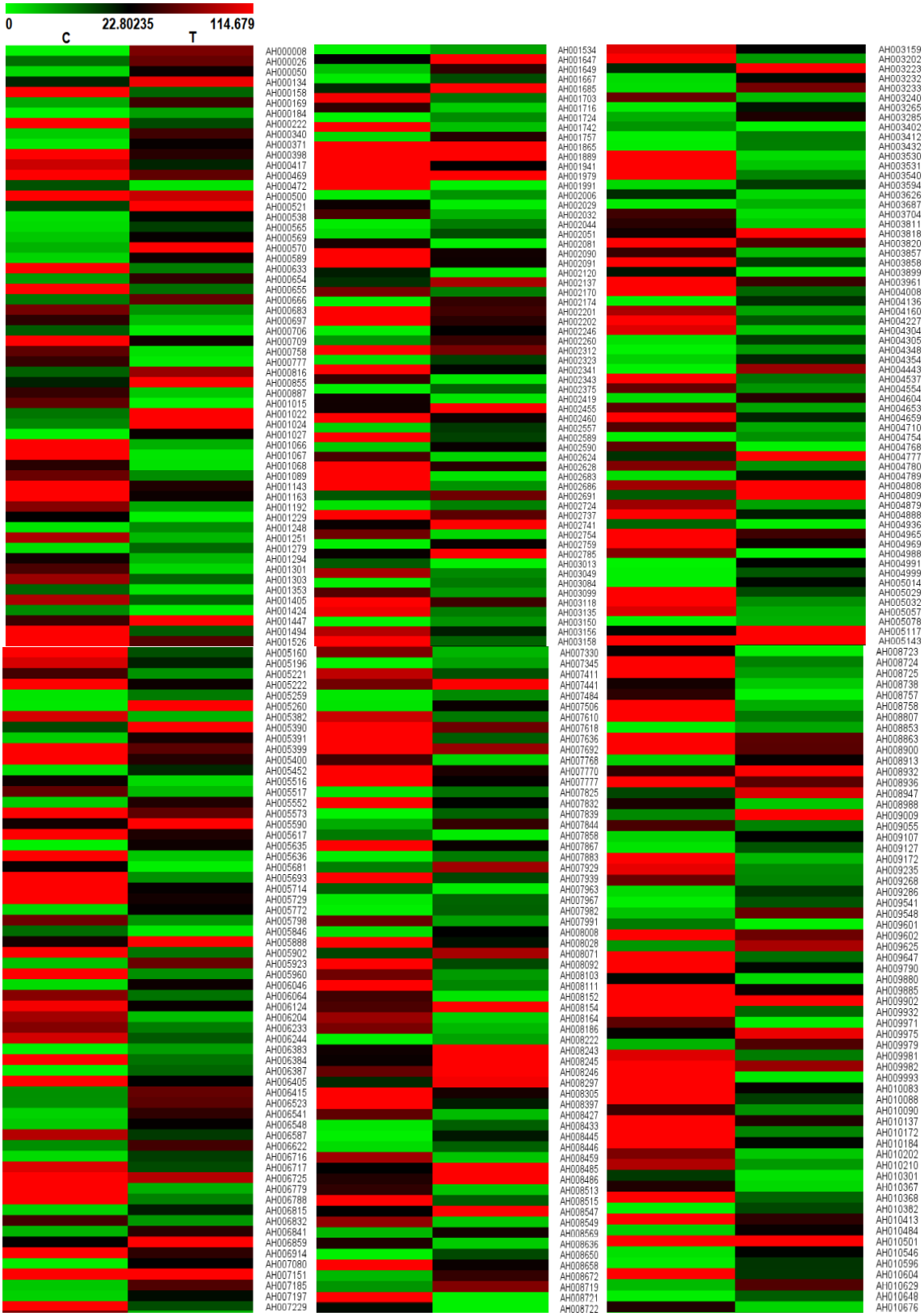


Figure 8.17: Volcano plot of the sample comparison


0
22.80235
114.679
C
T

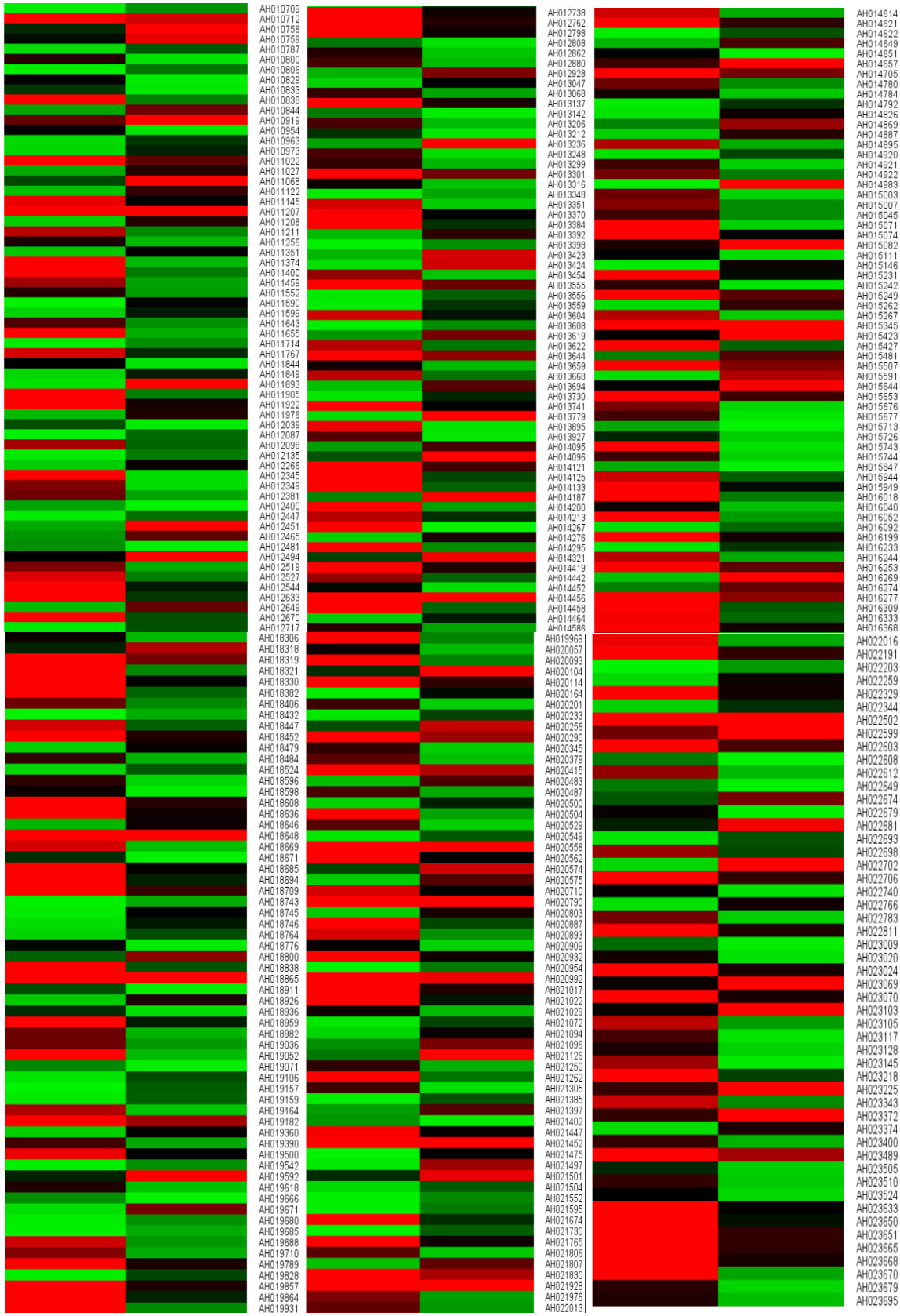


Figure 8.18: Heat map of DEGs

5. **Gene Ontology Analysis (GOA)-** All the transcripts are grouped into molecular, cellular and biological based on gene ontology analysis. The GO analysis revealed that the differentially expressed genes involved both in up and down regulation are part of important molecular functions like metal ion binding, transmembrane transport activity, oxidoreductase activity, DNA binding transcription factor activity. The majority of cellular process are cell membrane components and microtubule associated proteins. In the biological process category, the most enriched GO terms are transmembrane transport, metabolic processes, regulation of transcription and oxidation reduction processes. The bar plot of top 10 highly enriched GO terms in Up and Down-regulated genes of "C" vs. "T" comparison with p-value <= 0.05 were provided in figures 8.19 -8.25. The complete GO list is given below in table 8.6 – 8.11.

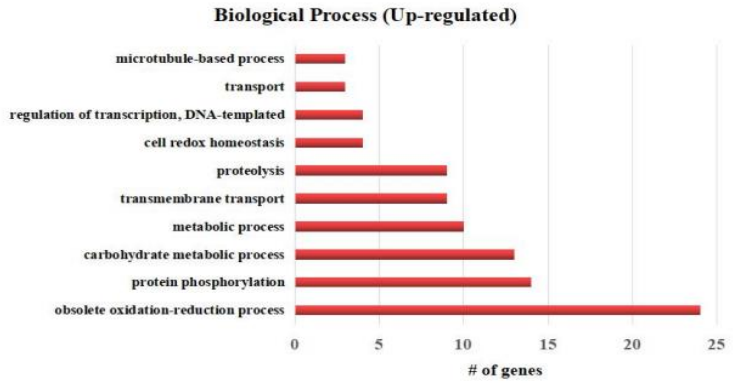


Figure 8.19: Top 10 Biological Processes (Up-regulated)

Table 8.6: Complete list of biological process (Up-regulated)

GO Term	Count
obsolete oxidation-reduction process	24
protein phosphorylation	14
carbohydrate metabolic process	13
metabolic process	10
transmembrane transport	9
Proteolysis	9
cell redox homeostasis	4
regulation of transcription, DNA-templated	4
Transport	3
microtubule-based process	3
lipid metabolic process	3
thiamine biosynthetic process	2
response to stress	2
Translation	2
regulation of transcription by RNA polymerase II	2
response to oxidative stress	2
aromatic compound catabolic process	1
biosynthetic process	1

molybdate ion transport	1
protein peptidyl-prolyl isomerization	1
cell wall modification	1
cation transport	1
aromatic amino acid family biosynthetic process	1
protein folding	1
Phosphorylation	1
tricarboxylic acid cycle	1
carbon fixation	1
protein ubiquitination	1
Photosynthesis	1
regulation of DNA-templated transcription, elongation	1
cellular glucose homeostasis	1
pentose-phosphate shunt	1
phospholipid transport	1
L-phenylalanine biosynthetic process	1
protein import	1
cell wall biogenesis	1
glycine catabolic process	1
carbohydrate phosphorylation	1
phosphorelay signal transduction system	1
protein catabolic process	1
folic acid-containing compound biosynthetic process	1
malate transport	1
nitrate assimilation	1
L-arabinose metabolic process	1
negative regulation of translation	1
signal transduction	1
translational initiation	1
photosynthesis, light harvesting	1
Mo-molybdopterin cofactor biosynthetic process	1
glucose metabolic process	1
steroid biosynthetic process	1
metal ion transport	1
response to hormone	1
obsolete pathogenesis	1

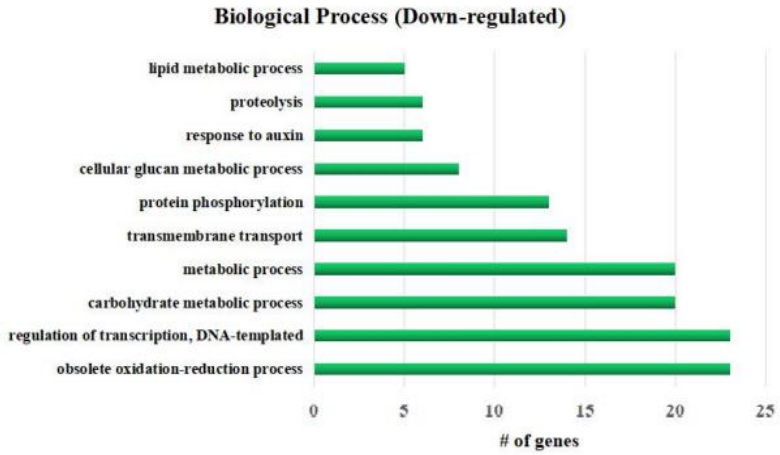


Figure 8.20: Top 10 Biological Processes (Down-regulated)

Table 8.7: Complete list of Biological Process (Down-regulated)

GO Term	Count
obsolete oxidation-reduction process	23
regulation of transcription, DNA-templated	23
carbohydrate metabolic process	20
metabolic process	20
transmembrane transport	14
protein phosphorylation	13
cellular glucan metabolic process	8
response to auxin	6
Proteolysis	6
lipid metabolic process	5
phosphorelay signal transduction system	4
response to oxidative stress	4
protein ubiquitination	4
cell redox homeostasis	4
protein dephosphorylation	3
xenobiotic transmembrane transport	3
Transport	3
metal ion transport	3
defense response	2
signal transduction	2
autophagosome assembly	2
glutamine metabolic process	2
iron ion transport	2
cellulose biosynthetic process	2
fatty acid biosynthetic process	2
cellular iron ion homeostasis	2
nucleotide biosynthetic process	1
magnesium ion transport	1
carboxylic acid metabolic process	1
nucleotide-excision repair	1
rRNA processing	1
double-strand break repair via homologous recombination	1
cellular ion homeostasis	1
mRNA processing	1
lipid biosynthetic process	1
small GTPase mediated signal transduction	1
carbohydrate phosphorylation	1
protein homo-oligomerization	1
negative regulation of translation	1
Translation	1
response to wounding	1
polysaccharide catabolic process	1
proteasome-mediated ubiquitin-dependent protein catabolic process	1
vesicle-mediated transport	1
translational initiation	1
phosphate-containing compound metabolic process	1
pyrimidine nucleotide biosynthetic process	1
cell growth	1
amino acid transmembrane transport	1
lipid glycosylation	1
glyoxylate cycle	1
response to sucrose	1
L-arabinose metabolic process	1
glycerol ether metabolic process	1
aromatic amino acid family metabolic process	1
tyrosine metabolic process	1
histidine biosynthetic process	1

regulation of cell cycle	1
vacuolar proton-transporting V-type ATPase complex assembly	1
cellular glucose homeostasis	1
cellular response to stimulus	1
cellulose microfibril organization	1
mitochondrial pyruvate transmembrane transport	1
cation transport	1
L-phenylalanine catabolic process	1
cytokinin metabolic process	1
peroxisome organization	1
protein import into mitochondrial matrix	1
inositol catabolic process	1
cellular response to nitrogen levels	1
protein folding	1
protein import into peroxisome matrix	1
biosynthetic process	1

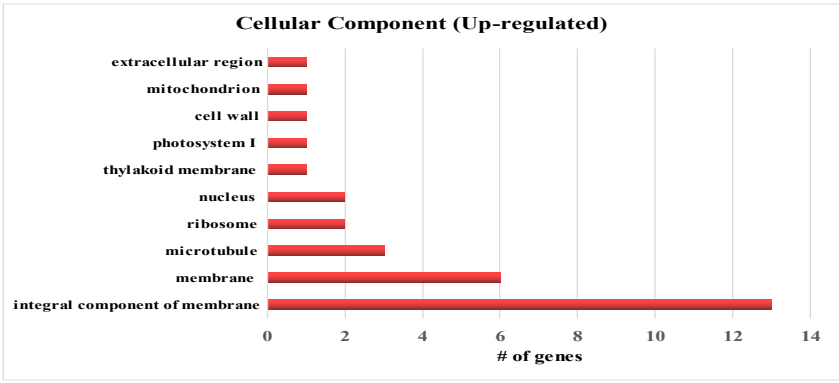


Figure 8.21: Top 10 Cellular Components (Up-regulated)

Table 8.8: Complete list of Cellular Process (Up-regulated)

GO Term	Count
integral component of membrane	13
Membrane	6
Microtubule	3
Ribosome	2
Nucleus	2
thylakoid membrane	1
photosystem I	1
cell wall	1
Mitochondrion	1
extracellular region	1
mediator complex	1

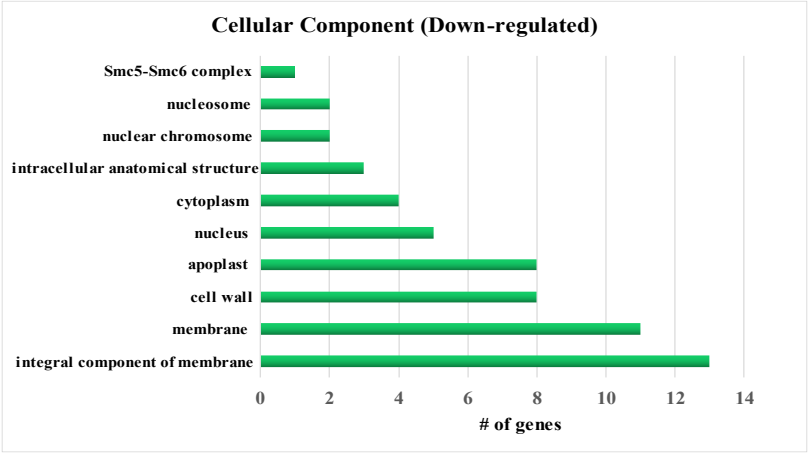


Figure 8.23: Top 10 Cellular Components (Up-regulated)

Table 8.9: Complete list of Cellular Process (Down-regulated)

GO Term	Count
integral component of membrane	13
Membrane	11
cell wall	8
Apoplast	8
Nucleus	5
Cytoplasm	4
intracellular anatomical structure	3
nuclear chromosome	2
Nucleosome	2
Smc5-Smc6 complex	1
mitochondrial inner membrane	1
anaphase-promoting complex	1
Ribosome	1
integral component of peroxisomal membrane	1
THO complex part of transcription export complex	1
anchored component of membrane	1
TIM23 mitochondrial import inner membrane translocase complex	1
small-subunit processome	1
anchored component of plasma membrane	1

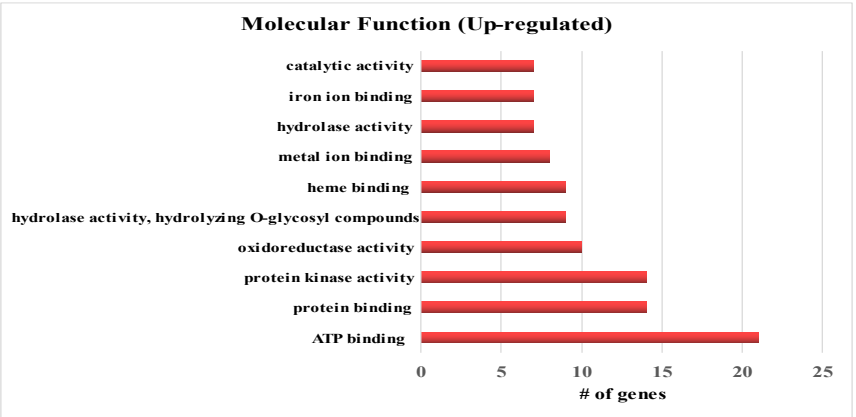


Figure 8.24: Top 10 Molecular Functions (Up-regulated)

Table 8.10: Complete list of Molecular Functions (Up-regulated)

GO Term	Count
ATP binding	21
protein binding	14
protein kinase activity	14
oxidoreductase activity	10
hydrolase activity, hydrolysing O-glycosyl compounds	9
heme binding	9
metal ion binding	8
hydrolase activity	7
iron ion binding	7
catalytic activity	7
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	6
DNA binding	6
serine-type endopeptidase activity	4
methyltransferase activity	4
ATPase-coupled transmembrane transporter activity	3
RNA binding	3
ATP hydrolysis activity	3
iron-sulfur cluster binding	3
calmodulin binding	3
GTPase activity	3
hydrolase activity, acting on ester bonds	3
nucleic acid binding	3
flavin adenine dinucleotide binding	3
endopeptidase inhibitor activity	3
cysteine-type peptidase activity	3
acyltransferase activity, transferring groups other than amino-acyl groups	3
2 iron, 2 sulfur cluster binding	2
ubiquitin-protein transferase activity	2
transmembrane transporter activity	2
cation binding	2
peroxidase activity	2
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	2
NADP binding	2
phosphotransferase activity, alcohol group as acceptor	2
hexosyltransferase activity	2
enzyme inhibitor activity	2
zinc ion binding	2
electron transfer activity	2
carbohydrate binding	2
structural constituent of ribosome	2
starch binding	2
transcription coregulator activity	1
phosphoenolpyruvate carboxylase activity	1
sulfotransferase activity	1
molybdenum ion binding	1
chlorophyllide a oxygenase	1
glycosyltransferase activity	1
protein-disulfidereductase activity	1
phosphogluconate dehydrogenase (decarboxylating) activity	1
peptidyl-prolylcis-trans isomerase activity	1
Tetrahydrofolyl polyglutamate synthase activity	1
glucose binding	1
alpha-L-arabinofuranosidase activity	1
polygalacturonase activity	1

ATPase-coupled intramembrane lipid transporter activity	1
galactoside 2-alpha-L-fucosyltransferase activity	1
pectinesterase activity	1
double-stranded DNA binding	1
3-deoxy-7-phosphoheptulonate synthase activity	1
kinase activity	1
glycine dehydrogenase (decarboxylating) activity	1
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H asone donor, and incorporation of two atoms of oxygen into one donor	1
molybdate ion transmembrane transporter activity	1
DNA-binding transcription factor activity	1
oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	1
carbon-nitrogen ligase activity, with glutamine as amido-N-donor	1
magnesium ion binding	1
NAD binding	1
4-alpha-glucanotransferase activity	1
ADP binding	1
cationtransmembrane transporter activity	1
rRNA N-glycosylase activity	1
serine-type endopeptidase inhibitor activity	1
guanyl-nucleotide exchange factor activity	1
hexokinase activity	1
3-beta-hydroxy-delta5-steroid dehydrogenase activity	1
translation initiation factor activity	1
ATP-dependent peptidase activity	1
catechol oxidase activity	1
sulfateadenylyltransferase (ATP) activity	1
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	1
glucose-6-phosphate dehydrogenase activity	1
amino acid binding	1
nucleotide binding	1
lipid binding	1
protein dimerization activity	1
prephenatedehydratase activity	1
transferase activity	1
sequence-specific DNA binding	1
aspartic-type endopeptidase activity	1
serine-type carboxypeptidase activity	1

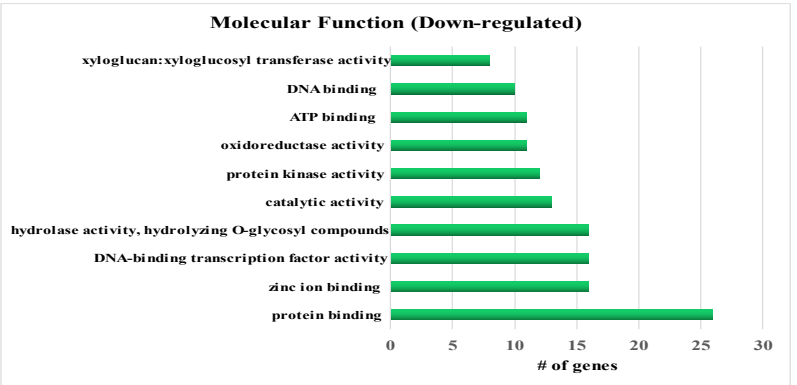


Figure 8.25: Top 10 Molecular Functions (Down-regulated)

Table 8.11: Complete list of Molecular Functions (Down-regulated)

GO Term	Count
protein binding	26
zinc ion binding	16
DNA-binding transcription factor activity	16
hydrolase activity, hydrolysing O-glycosyl compounds	16
catalytic activity	13
protein kinase activity	12
oxidoreductase activity	11
ATP binding	11
DNA binding	10
xyloglucan:xyloglucosyltransferase activity	8
heme binding	7
metal ion binding	6
aspartic-type endopeptidase activity	5
sequence-specific DNA binding	5
nucleic acid binding	4
electron transfer activity	4
iron ion binding	4
ubiquitin-protein transferase activity	4
protein-disulfidereductase activity	4
hydrolase activity	4
acyltransferase activity, transferring groups other than amino-acyl groups	4
peroxidase activity	4
xenobiotic transmembrane transporter activity	3
hexosyltransferase activity	3
transmembrane transporter activity	3
antiporter activity	3
calcium ion binding	3
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	3
protein serine/threonine phosphatase activity	3
obsolete coenzyme binding	3
amino acid binding	2
phosphoric diester hydrolase activity	2
endopeptidase inhibitor activity	2
magnesium ion binding	2
cellulose synthase (UDP-forming) activity	2
pyridoxal phosphate binding	2
oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	2
ferric iron binding	2
nutrient reservoir activity	2
transporter activity	2
flavin adenine dinucleotide binding	2
phosphotransferase activity, alcohol group as acceptor	2
GTP binding	1
acyl-CoA dehydrogenase activity	1
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	1
hexokinase activity	1
hydrolase activity, acting on ester bonds	1

homogentisate 1,2-dioxygenase activity	1
chaperone binding	1
carbohydrate binding	1
sulfotransferase activity	1
calcium-dependent phospholipid binding	1
glucose binding	1
serine-type endopeptidase activity	1
beta-amylase activity	1
oxidoreductase activity, acting on the CH-CH group of donors	1
NADH dehydrogenase (ubiquinone) activity	1
translation initiation factor activity	1
enzyme inhibitor activity	1
nucleotide binding	1
CTP synthase activity	1
acyltransferase activity	1
folic acid binding	1
fatty-acyl-CoA binding	1
damaged DNA binding	1
copper ion binding	1
thiolester hydrolase activity	1
amino acid transmembrane transporter activity	1
structural constituent of ribosome	1
alpha-L-arabinofuranosidase activity	1
inositol oxygenase activity	1
cyclin-dependent protein serine/threonine kinase inhibitor activity	1
cation binding	1
voltage-gated anion channel activity	1
protein serine/threonine kinase activity	1
triglyceride lipase activity	1
ATPase activator activity	1
omega peptidase activity	1
ribose phosphate diphosphokinase activity	1
rRNA N-glycosylase activity	1
glycosyltransferase activity	1
nitronatemonooxygenase activity	1
cytokinin dehydrogenase activity	1
inorganic diphosphatase activity	1
serine-type endopeptidase inhibitor activity	1
transferase activity	1
solute:protonantiporter activity	1
acid phosphatase activity	1
protein domain specific binding	1
isocitratelyase activity	1
malate synthase activity	1
phosphoribosyl-AMP cyclohydrolase activity	1
4-hydroxyphenylpyruvate dioxygenase activity	1
carbon-sulfurlyase activity	1
magnesium ion transmembrane transporter activity	1
transaminase activity	1
NAD binding	1
N-acetyltransferase activity	1
SUMO transferase activity	1

Study on selected gene response to Cr^{VI} stress

Differentially expressed heavy metal transporters due to Cr^{VI} stress

There are many identified transporters involved in heavy metal uptake and transport that play a vital role in Cr^{VI} induced stress. In the present study, heavy metal transporter families like ATP binding cassette transporter, V-Type ATPase, ZIP are the identified DEGs, which are seen significantly differentially expressed in *Amaranthus dubius* under 70mg Cr^{VI} stressed condition. Heavy metal homeostasis is maintained by vacuolar sequestration and is driven by V-type ATPases and tonoplast transporters (Sharma *et al.*, 2016). Vacuolar ATPases with gene ID AH009055 is expressed to be downregulated by 2-fold indicating an obvious non-functional molecular transport mechanism due to heavy metal stress. Other transmembrane proteins like branched chain amino acid transaminase-2, cationic amino acid transporter 2 are also seen downregulated in the present study. MATE efflux family proteins are transmembrane proteins which are seen downregulated by 4.2-fold (AH002029, AH012349, AH015007) due to Cr^{VI} induced stress in 70mg treated plants. The MATE proteins are located in plasma membrane and vacuole, which transport secondary metabolites through H+ exchange that play a crucial role in eliminating endotoxins and exotoxins, especially heavy metals (Shoji, 2014). The downregulation of four MATE efflux family protein genes suggest that Cr^{VI} toxicity hindered all molecular mechanism behind heavy metal transport and elimination in the highest concentration of Cr^{VI} treated *A. dubius*.

ATPase coupled transmembrane (Multi drug resistance protein) with gene ID AH002006, AH017339, AH02928) are seen upregulated by 2 to 3-fold when exposed to 70 mg Cr^{VI} compared to control in *A. dubius*. Various types of substrates like drugs, metal ions, lipids and steroids are transported across plasma membrane, intra cellular membrane through ABC transporters. The result suggested that ABC transporters has a crucial role in the transport of Cr^{VI} into the cell along with metal ion transporters. AH015481, AH018596 and AH012098 are heavy metal transport and detoxification associated genes in which two are downregulated by 2 to 3-fold whereas one with gene ID AH015481 is upregulated by 2-fold. Two genes with gene IDAH008650 are (HMA-2) Heavy Metal ATPase 2 that are upregulated by 3.5-fold. The engulfed metal ions are transported from

cytosol to the vacuole for sequestration via transporters. The RNA sequence study of *A. dubius* with Cr^{VI} treatment reveals the up regulation of ATHMA2, HMA2 3.6-fold compared to control. Hazan *et al.* (2017) reported that, RNA-Seq and de novo transcriptome analysis of different candidate genes that encode heavy metal ATPases (HMAs), ABC transporter, zinc iron permeases (ZIPs) and natural resistance-associated macrophage proteins (NRAMPs) are involved in metal transport and cellular detoxification (Xu *et al*., 2015; Sharma *et al*., 2016).

Differentially expressed metal ion binding genes in response to Cr^{VI} stress

The previous report that the alterations of iron related pathways as an association between concentrations of Fe and other metals like Cr, As, Cd together with alteration in expression of genes involved in iron homeostasis (Mackenzie *et al*., 2008). In this respect, the present study observations suggests that, the iron handling pathways play an important role in the response of *Amaranthus dubius* to Cr^{VI} metal exposure. CHY-type/CTCHY-type/RING-type (AH001229) and zinc ion binding (AH019182 and AH022740), copper-exporting ATPase / responsive-to-antagonist 1 / copper-transporting ATPase (AH012808) are also downregulated by 2-fold. Enhanced expression of molybdenum ion binding with 4.5-fold up regulation is observed in 70mg Cr^{VI} metal stressed plant in contradiction to other metal ion transporting mechanisms.

Differentially expressed antioxidant enzymes genes in response to Cr^{VI} stress

Excess accumulation of Cr^{VI} induces reactive oxygen species (ROS) production creating oxidative damages to DNA, RNA, protein, lipids and cells, which enhances the malondialdehyde (MDA) content leading to plant growth retardation. During the initial level stress treatments of Cr^{VI}, ROS production is minimalized by increasing antioxidant enzymes like SOD, CAT, POD and compounds like ascorbate and glutathione. In the physio-biochemical studies carried out in the chapter 3, there is a decreasing trend in the antioxidant enzyme activities inferred in *Amaranthus dubius* plants treated with higher concentrations of Cr^{VI} stress and these inferences are supported by the down regulation trend in the gene expression of SOD, CAT, thioredoxin super family proteins recorded in this chapter with respect to 70mg Cr^{VI} stressed *A. dubius* plants. The genes of superoxide dismutase with gene ID AH019268, AH001228, AH006896, AH007534 AH023731 and

AH023733 are seen down regulated by 0.9-fold and catalase genes with gene ID AH023731, AH023733 by 1.5-fold. A very few genes upregulated are AH018780 and AH011513 of SOD which indicates a highly efficient antioxidant system in *A. dubius* which is disrupted gradually by increasing Cr^{VI} stress.

Many genes related to peroxidase super family proteins (AH001424, AH003820, AH006244, AH013236, AH021501, AH021976) are seen downregulated during 70mg Cr^{VI} stress. Chromium stress cause lipid peroxidation of thylakoid membrane and the downregulation of peroxidases may be due to Cr^{VI} toxicity induced damage by lipid peroxidation of thylakoid membrane by lipoxygenases (Chakrabarty *et al.*, 2009). The inference obtained with respect to antioxidant enzyme activities in the previous chapter of physio-biochemical studies are in support of the above view, in which the chlorophyll pigments are seen reduced quantitatively suggesting the disruption of chloroplast ultrastructure and inhibition of electron transport chain due to higher Cr^{VI} stress. Previous studies have revealed that high concentration of glutathione could form unstable complex with Cr^{VI} to form Cr^{VI} GSH, which in turn is sequestered into vacuole by the catalytic action of GST thereby reducing cell damage in plants (Levina *et.al*, 2003). Under Cr^{VI} stress, 14 genes (represented in heat map) defining Glutathione S Transferase family protein is found upregulated by 3 to 4-fold in the present study whereas 15 genes are found downregulated showing indications of disruption in the functioning of antioxidant defense mechanism.

Differentially expressed transcription factors related to Cr^{VI} stress

Transcription factors regulate abiotic stress genes especially under heavy metal stress and are identified to be involved in downstream processing related to Cr^{VI} induced stress. The transcriptomic analysis done in Kenaf by Peng *et al*. (2021) revealed that many transcription factors like C2H2, NAC and WRKY families are expressed under Cd stress. The present study in *Amaranthus dubius*, most of the transcription factors, WRKY(AH0006555), C2H2 (AH001229), NAC (AH010413, AH008092, AH010413, AH017564, AH005222, AH005032), ARF (AH007839), ERF (AH009885, AH010083, AH021674, AH011400, AH018319) are seen downregulated by 4 to 3-fold under Cr^{VI} exposure whereas, the jasmonate–zim-domain protein 1(AH013392) is seen upregulated

by 2.7- fold. bHLH DNA binding super family protein is upregulated by 2 to 6-fold. All these observations in the study indicate that obvious complex regulatory pathway mechanisms are involved in the Cr^{VI} stressed *A. dubius* plants. Auxin response factor (ARF) is one of the two among auxin regulators involved in auxin signalling. In the current study, all the ARFs are seen downregulated in the Cr stress response pathways. Mitogen-activated protein kinase (MAPK) signalling pathway is affected by the heavy metal stress, initiates many transcription factors that regulate many signal transduction pathways of phytohormones. MAPK is observed to be downregulated by 2 to 5-fold.

Differentially expressed cell wall related genes to Cr^{VI} stress

Expansins is a large super family of proteins which code for cell wall proteins. With respect to expansins, it is observed that 4 genes (AH018319, AHO10709, AH016417, AH013694, AH000340) are upregulated and 2 genes downregulated (AH002341, AH000758) in *Amaranthus dubius*. Xyloglucan endotransglucosylase is part of hydrolase family, involved in the metabolism of xyloglucan that helps in the formation of cell wall and rapid cell expansion and in this respect, eight genes with gene ID AH020558, AH020562, AH011552, AH018452, AH008757, AH015743, AH008758, AH004768 is seen downregulated by 2 to 5-fold.

Response of Heat shock stress proteins (HSPs) to Cr^{VI} stress

HSPs are expressed in all organisms in response to abiotic stress which assist in the folding and translocations of other proteins. High exposure to Cr treatment has reported the expressions of HSP90-1 in tomato plants. Sun *et al*. (2001) in their studies characterized the gene that encoded the cytosolic class II smHSP in *Arabidopsis thaliana* (At-HSP17.6A). The At-HSP17.6A expression was induced by heat and osmotic stress, as well as during seed development. 17.6 KDa heat shock proteins with gene ID (AH016052) and Hsp20 (AH013668, AH023024) like chaperone proteins are seen downregulated by 2 to 5-fold under Cr^{VI} stress. The heat shock proteins (HSPs) function as surveillance mechanisms, which are preferentially expressed under stress to maintain functional and healthy proteomes (Amm *et al*., 2014; Hazan *et al*., 2017). The present study shows an up regulation of 6-fold DNA J heat shock protein in 70mg Cr^{VI} treated plants compared to the control in *Amaranthus dubius* plants.

Cytp450

In the transcriptomic studies conducted in rice, large number of Cytochrome p450 related genes are seen upregulated during heavy metal Cr^{VI} stress indicating their role in metal detoxification (Rai *et al*., 2015). With respect to *A. dubius* in the present study, the cytp450 related genes with gene ID AH003232, AH003233, AH007929, AH021397, AH016607 are seen upregulated whereas two genes AH018776 and AH015074 are downregulated in 70mg Cr^{VI} treatment which clearly indicates the response of cytp450 under oxidative stress and toxicity.

Calmodulin

In the case of Calmodulin binding family protein (AH006716, AH014213, AH021595) two genes are seen upregulated and two are downregulated in *A. dubius* plants treated with 70mg Cr^{VI} stress. Calmodulin are great sensors of heavy metals which are triggered by the induction of Ca^{2+}ions and changes their conformations on binding to Ca^{2+} inside the cells in response to external stimuli or abiotic stress, regulate ion transport and stress tolerance (DalCorso, 2010).

Stress related genes

Pathogenesis/disease related thaumatin super family genes upregulated to 6.5 times under 70mg Cr^{VI} stress than the control in *Amaranthus dubius*. The Cr^{VI} stress stimulated the expression of JA, SA and ethylene and this suggests crucial cross talks are carried out between Cr^{VI} stress and heavy metal signalling pathways in *A. dubius*. Plants under metal stress are reported to synthesize small Cysteine-rich oligomers called Phytochelatins (Ashraf *et al*., 2010; Pochodylo & Aristilde, 2017). In the present study, a 3.4-fold upregulation in CAP superfamily protein (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) is recorded in *A. dubius* under 70mg Cr^{VI} stress treatment.

Proline

Proline rich protein 4 is seen upregulated by 19-fold in 70mg Cr^{VI} stressed *A. dubius* compared to the control plant in the study. This clearly indicating the higher expression level of Proline rich protein 4 due to high oxidative stress induced by metal Cr^{VI}.

Response of Photosystems to Cr^{VI} stress

The transcriptome analysis in *Amaranthus dubius* reveals that many genes belonging to Light Harvesting Complexes Chlorophyll A-B binding protein family (*lhcb1*) seen to have up regulated from 0.9 to 2.3- fold while four genes belonging to light harvesting complexes PSI and PSII (*lhca5, lhcb5, lhca1, lhca6*) seen to have down regulated due to 70 mg Cr^{VI} stress. This indicates that Light Harvesting Photosystem I and Photosystem II are affected by oxidative stress due to metal Cr^{VI} and these observations are in agreement with the observations and inferences of the present study done in the chapter 3 with respect to photosynthetic rate, primary metabolite production etc.

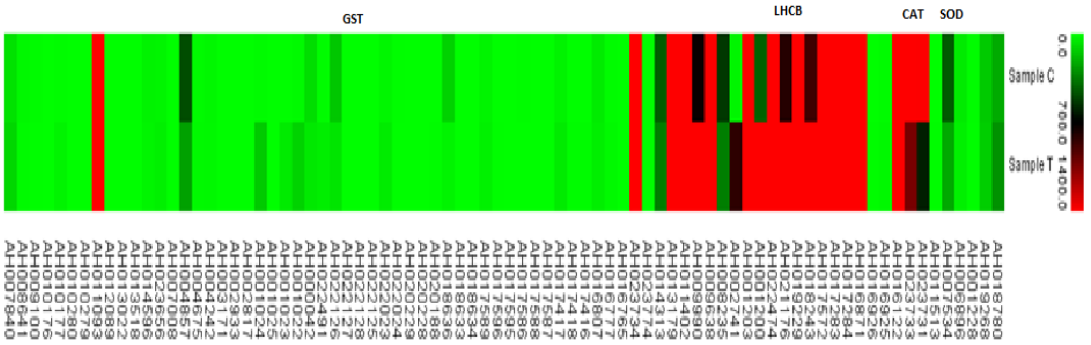


Figure 8.26: Heat map of selected DEGs under Cr^{VI} stress. Red colour indicates higher expression and green colour the least expression of genes. SOD- Super Oxide Dismutase, CAT- Catalase, LHCB – Light Harvesting Chlorophyll Binding Protein, GST – Glutathione -S- Transferase

Real-Time / qPCR Analysis

Four differentially expressed genes SOD, POD, LHCB1, GSTU21 are selected for validation of gene expression analysis in *Amaranthus dubius* and the expression level is quantified by qRT-PCR. CQ values obtained, mean CQ, standard deviation, PCR efficiency, and fold of induction of BTUB (House-Keeping Gene- table 8.12 & 8.13 and figure 8.26a & 8.27), *Superoxide dismutase (*SOD- table 8.14, 8.15 & 8.16 and figure 8.28 & 8.29), Catalase (CAT- table 8.18, 8.19 & 8.20 and figure 8.31 & 8.32), Light-harvesting chlorophyll a/b-binding proteins (LHCB1- table 8.22, 8.23 & 8.24 and figure 8.34 & 8.35) and Glutathione S-transferases (GSTU21- table 8.26, 8.27 & 8.28 and figure 8.37 & 8.38) genes are given below. Standard deviations are below 0.2 which are permissible for the reliability of results. The melt curve and melt peak figures are attached.

BTUB (house-keeping gene)

Table 8.12: CQ value of BTUB

Gene Name	Tube NAME	CQ value	Mean CQ	Std. deviation
BTUB	C1	6.61	6.61	0.169706
BTUB	C1	6.49		
BTUB	C1	6.73		
BTUB	T1	7.32	7.215	0.105
BTUB	T1	7.11		
BTUB	T1	7.215		
BTUB	C5	6.61	6.61	0.169706
BTUB	C5	6.49		
BTUB	C5	6.73		
BTUB	T5	7.58	7.58	0.27
BTUB	T5	7.85		
BTUB	T5	7.31		
BTUB	C10	6.61	6.61	0.169706
BTUB	C10	6.49		
BTUB	C10	6.73		
BTUB	T10	19.35	19.55	0.2
BTUB	T10	19.75		
BTUB	T10	19.55		
BTUB	C20	6.61	6.61	0.169706
BTUB	C20	6.49		
BTUB	C20	6.73		
BTUB	T20	19.01	19.01333	0.095044
BTUB	T20	19.11		
BTUB	T20	18.92		
BTUB	C30	6.61	6.61	0.169706
BTUB	C30	6.49		
BTUB	C30	6.73		
BTUB	T30	7.33	7.33	0.02
BTUB	T30	7.35		
BTUB	T30	7.31		
BTUB	C50	6.61	7.129	0.169706
BTUB	C50	6.49		
BTUB	C50	6.73		
BTUB	T50	7.475	7.475	0.245
BTUB	T50	7.23		
BTUB	T50	7.72		

Table 8.13: PCR efficiency of BTUB

			cycle #
	cDNA input		crossing threshold
dilution	**cDNA amount**	lncDNA	**Ct (or CP)**
1x	1	0	8.40
3x	0.333333333	-1.09861229	16.96
9x	0.111111111	-2.19722458	17.70
linear regr. of Ct / ln(DNA):		K	-4.232612
		D	9.703333
		y = kx + d	
slope (calc. for 10x dil.)		-9.7460	
PCR efficiency		**1.2665**	

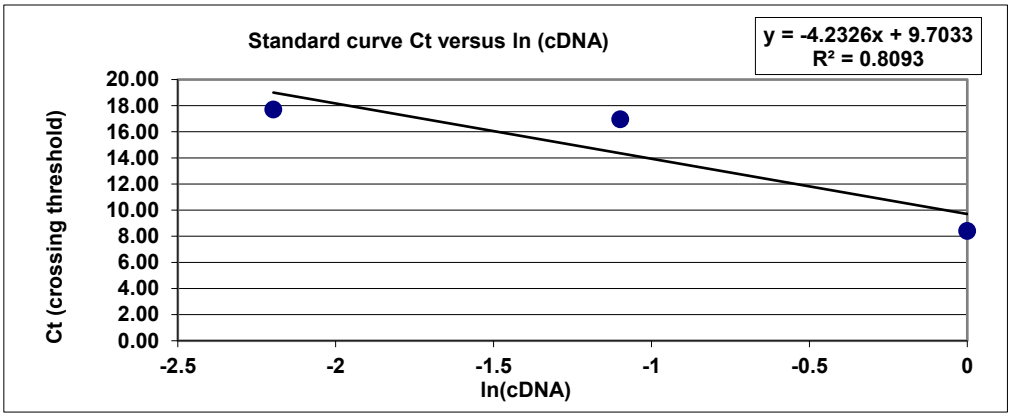


Figure 8.26a: Standard curve versus in (cDNA) for BTUB

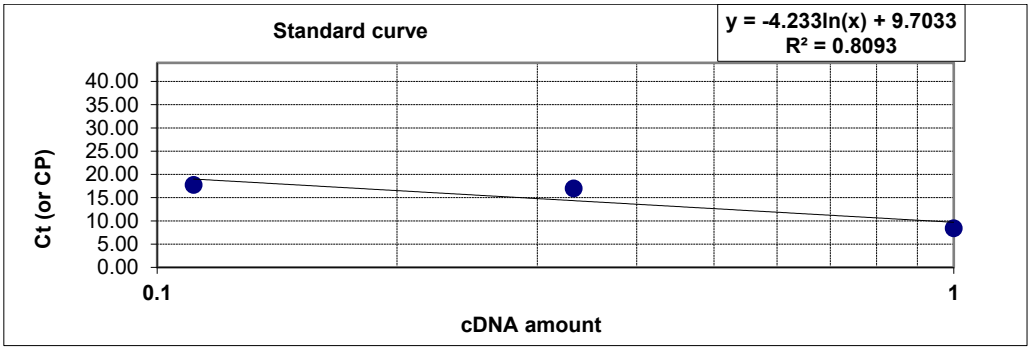


Figure 8.27: Standard curve of BTUB

***Superoxide dismutase (*SOD)**

SOD belongs to the family of metalloenzymes which acts as the primary line of defense against ROS-induced damages. The SOD catalyzes the removal of O•−2 by dismutation it into O2 and H2O2. Li *et al*. (2018) reported that there is an increase in expression level and mRNA transcripts of antioxidant enzymes like SOD, CAT, GST and ascorbate peroxidase when treated with chromium in studies conducted on *Brassica napus L*. In this study the fold of induction of SOD activity increases to a maximum at 20 mg chromium treatment with a value of 4078.11 and decreases till 50 mg treatment substantiate the evidences obtained in RNA sequence studies as well as those in biochemical studies (table 8.17 and figure 8.30).

Table 8.14: CQ value of SOD

Gene Name	Tube Name	CQ value	Mean CQ	Std deviation
SOD	C1	25.92	25.67	0.226274
SOD	C1	25.83		
SOD	C1	25.51		
SOD	T1	26.405	26.405	0.095
SOD	T1	26.5		
SOD	T1	26.31		
SOD	C5	25.92	25.670	0.226274
SOD	C5	25.83		
SOD	C5	25.51		
SOD	T5	26.93	26.873	0.049329
SOD	T5	26.84		
SOD	T5	26.85		
SOD	C10	25.92	25.670	0.226274
SOD	C10	25.83		
SOD	C10	25.51		
SOD	T10	16.09	16.090	0.11
SOD	T10	16.2		
SOD	T10	15.98		
SOD	C20	25.92	25.670	0.226274
SOD	C20	25.83		
SOD	C20	25.51		
SOD	T20	13.29	13.290	0.01
SOD	T20	13.28		
SOD	T20	13.3		
SOD	C30	25.92	25.670	0.226274
SOD	C30	25.83		
SOD	C30	25.51		
SOD	T30	13.84	13.940	0.1
SOD	T30	14.04		
SOD	T30	13.94		
SOD	C50	25.92	25.670	0.226274
SOD	C50	25.83		
SOD	C50	25.51		
SOD	T50	13.93	13.597	0.308923
SOD	T50	13.54		
SOD	T50	13.32		

Table 8.15: The PCR efficiency of SOD

			cycle #
	cDNA input		crossing threshold
dilution	**cDNA amount**	lncDNA	**Ct (or CP)**
1x	1	0	11.86
3x	0.333333333	-1.09861229	14.23
9x	0.111111111	-2.19722458	15.84
linear regr. Of Ct / ln(DNA):		k	-1.811376
		d	11.986667
		y = kx + d	
slope (calc. for 10x dil.)			-4.1708
PCR efficiency			**1.7368**

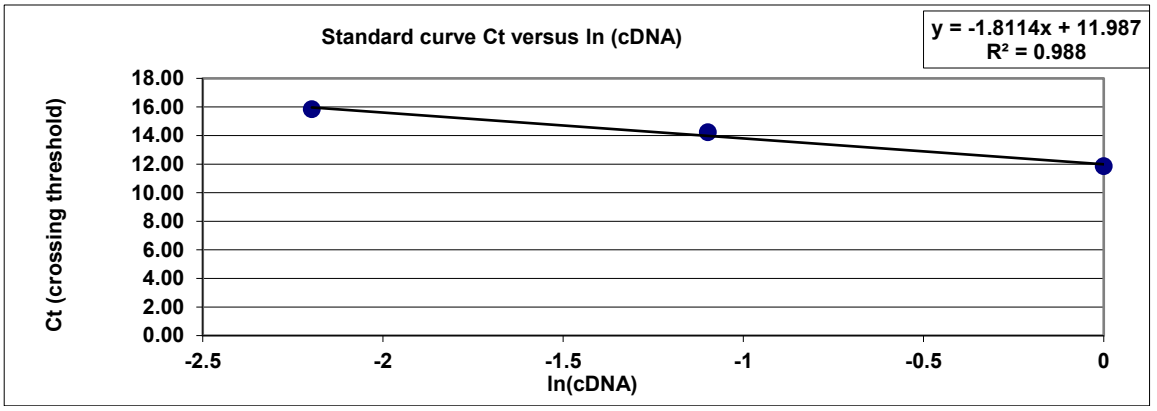


Figure 8.28: Standard curve versus in (cDNA) for SOD

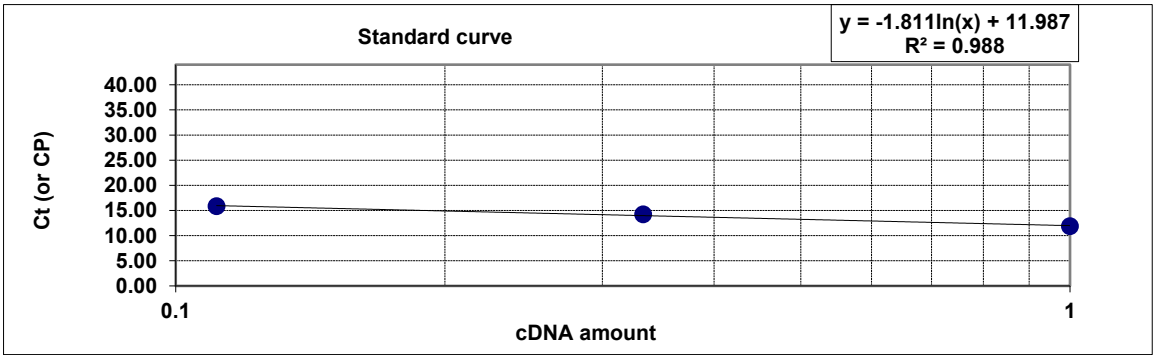


Figure 8.29: Standard Curve of SOD

Table 8.16: The fold of induction of SOD

	target gene	reference gene	
PCR efficiency	1.7368	1.1267	
Ct values	**Ct target**	**Ct reference**	**fold induction**
control	25.670	6.61	1
1mg	26.405	7.215	0.716335125
5mg	26.873	7.58	0.577851611
10mg	16.090	19.55	926.729774
20mg	13.290	19.01333	4078.110335
30mg	13.940	7.33	707.2181074
50mg	13.597	7.475	869.5548159

Table 8.17: Mean Cq and Fold Induction of SOD Gene.

Sample	Gene	Mean Cq	Fold of Induction
C	SOD	25.670	1
1 mg	SOD	26.405	0.716335125
5 mg	SOD	26.873	0.577851611
10 mg	SOD	16.090	926.729774
20 mg	SOD	13.290	4078.110335
30 mg	SOD	13.940	707.2181074
50 mg	SOD	13.597	869.5548159

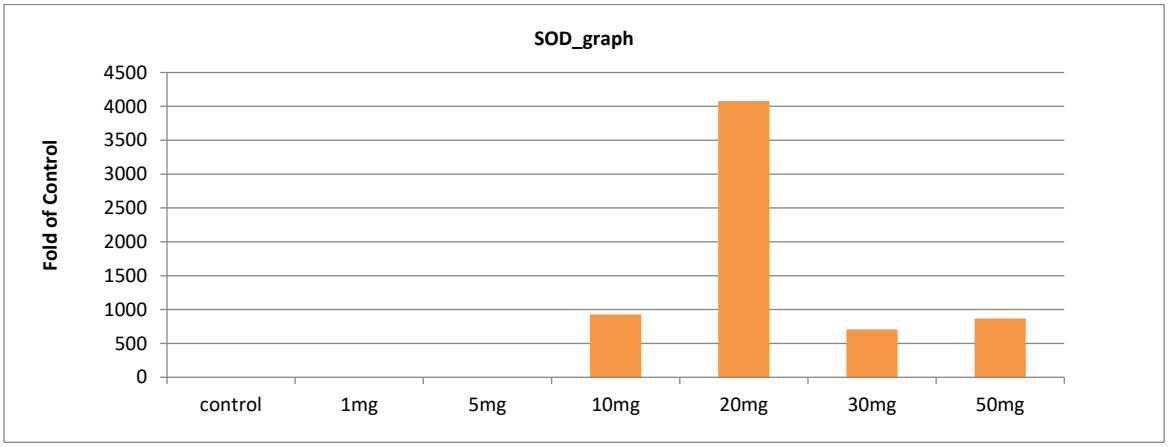


Figure 8.30: Fold Induction of SOD.

Catalase (CAT)

The antioxidant enzyme catalase eliminates H_2O_2 arising due to abiotic stress into water and oxygen. Leung (2018) has reported the expression of catalase at high level due to abiotic stresses like salinity, cold and H_2O_2 production arising due to heavy metal stress. Catalase activity was increased to maximum at 10 mg concentration and 20 mg Chromium treatment with a fold of induction of 10.39 and 11.75 respectively and later the activity decreased to a fold of induction of 1.52 (table 8.21and figure 8.33).

Table 8.18: CQ values of CAT

Gene Name	Tube Name	CQ value	MEAN CQ	Std. deviation
CAT	C1	17.00	17.195	0.106066
CAT	C1	17.27		
CAT	C1	17.12		
CAT	T1	17.24	17.50	0.253689
CAT	T1	17.53		
CAT	T1	17.74		
CAT	C5	17.00	17.195	0.106066
CAT	C5	17.27		
CAT	C5	17.12		
CAT	T5	19.45	19.78	0.292161
CAT	T5	19.99		
CAT	T5	19.90		
CAT	C10	17.00	17.195	0.106066
CAT	C10	17.27		
CAT	C10	17.12		
CAT	T10	11.50	11.67	0.246644
CAT	T10	11.95		
CAT	T10	11.55		
CAT	C20	17.00	17.195	0.106066
CAT	C20	17.27		
CAT	C20	17.12		
CAT	T20	10.25	10.38	0.132508
CAT	T20	10.51		
CAT	T20	10.38		
CAT	C30	17.00	17.195	0.106066
CAT	C30	17.27		
CAT	C30	17.12		
CAT	T30	17.62	17.62	0.35
CAT	T30	17.27		
CAT	T30	17.97		
CAT	C50	17.00	17.195	0.106066
CAT	C50	17.27		
CAT	C50	17.12		
CAT	T50	15.01	15.01	0.155
CAT	T50	15.16		
CAT	T50	14.85		

Table 8.19: The PCR efficiency of CAT

			cycle #
	cDNA input		crossing threshold
dilution	**cDNA amount**	lncDNA	**Ct (or CP)**
1x	1	0	10.45
3x	0.333333333	-1.09861229	16.95
9x	0.111111111	-2.19722458	25.65
linear regr. of Ct / ln(DNA):		k	-6.917818
		d	10.083333
		y = kx + d	
slope (calc. for 10x dil.)			-15.9289
PCR efficiency			**1.1555**

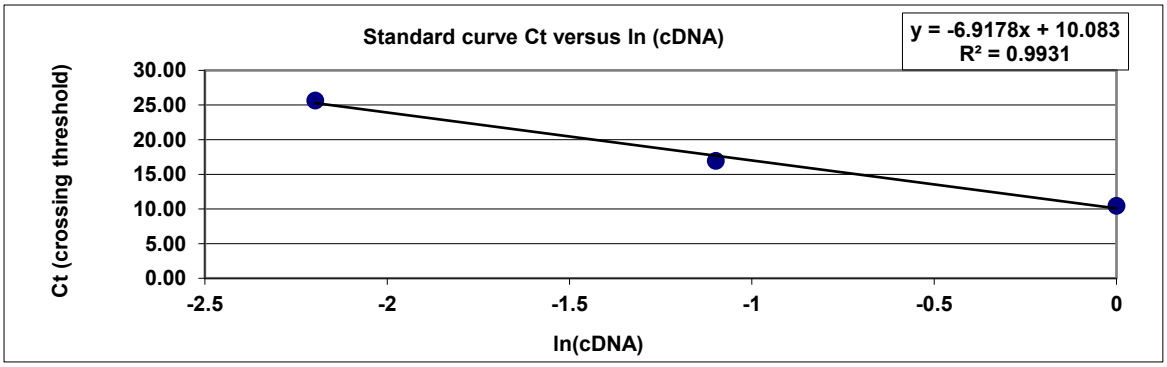


Figure 8.31: Standard curve versus in (cDNA) for CAT

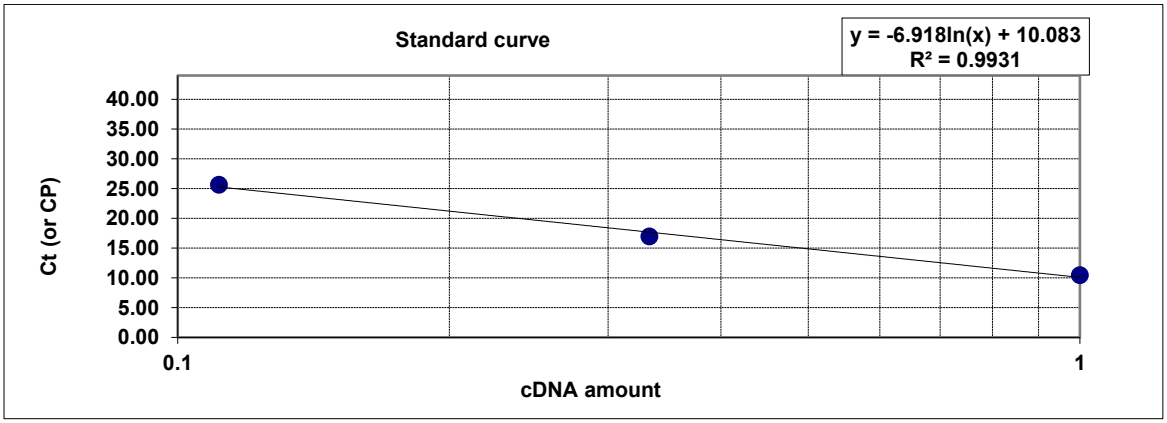


Figure 8.32: Standard curve of CAT

Table 8.20: The fold of induction of CAT

	Target gene	**Reference gene**	
PCR efficiency	1.1555	1.1267	
Ct values	**Ct target**	**Ct reference**	**fold induction**
control	17.195	6.61	1
1mg	17.5	7.215	1.028460279
5mg	19.78	7.58	0.772636161
10mg	11.67	19.55	10.39809797
20mg	10.38	19.01333	11.75258839
30mg	17.62	7.33	1.02473334
50mg	15.01	7.475	1.520371933

Table 8.21: Mean Cq and Fold Induction of CAT Gene.

Sample	**Gene**	**Mean Cq**	**Fold of Induction**
C	CAT	17.195	1
1 mg	CAT	17.5	1.028460279
5 mg	CAT	19.78	0.772636161
10 mg	CAT	11.67	10.39809797
20 mg	CAT	10.38	11.75258839
30 mg	CAT	17.62	1.02473334
50 mg	CAT	15.01	1.520371933

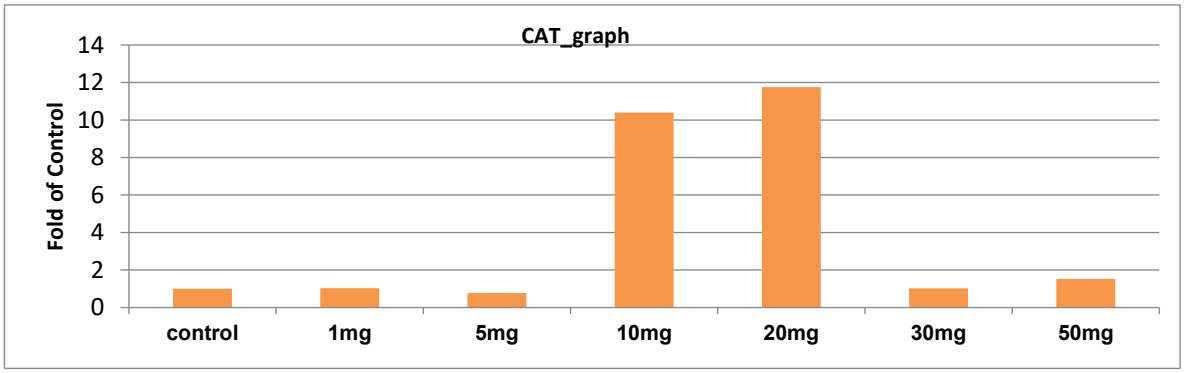


Figure 8.33: Fold Induction of CAT.

Light-harvesting chlorophyll a/b-binding proteins (LHCB1)

Light-harvesting chlorophyll a/b-binding proteins (LHCB) are apoprotein of light harvesting complex of Photosystem I and II seen associated with chlorophyll and xanthophyll (Jansson, 1994). Light-harvesting chlorophyll a/b-binding expression increased from 1 mg with a fold of induction of 464.24 which gradually decreases to 8.021 in 50 mg chromium treatment. It is observed to have a down regulation with increasing concentration of heavy metal stress (table 8.25 and figure 8.36).

Table 8.22: CQ values of Lhcb

Gene Name	Tube Name	CQ value	Mean CQ	Std. deviation
Lhcb1	C1	22.85	22.81	0.115326
Lhcb1	C1	22.68		
Lhcb1	C1	22.9		
Lhcb1	T1	9.93	10.08	1.37
Lhcb1	T1	10.18		
Lhcb1	T1	10.13		
Lhcb1	C5	22.85	22.81	0.115326
Lhcb1	C5	22.68		
Lhcb1	C5	22.9		
Lhcb1	T5	10.83	10.85	0.140475
Lhcb1	T5	10.85		
Lhcb1	T5	10.87		
Lhcb1	C10	22.85	22.81	0.115326
Lhcb1	C10	22.68		
Lhcb1	C10	22.9		
Lhcb1	T10	8.16	8.173333	0.02
Lhcb1	T10	8.32		
Lhcb1	T10	8.04		
Lhcb1	C20	22.85	22.81	0.115326
Lhcb1	C20	22.68		
Lhcb1	C20	22.9		
Lhcb1	T20	9.21	9.505	0.132288
Lhcb1	T20	9.505		
Lhcb1	T20	9.8		
Lhcb1	C30	22.85	22.81	0.115326
Lhcb1	C30	22.68		
Lhcb1	C30	22.9		
Lhcb1	T30	13.32	13.19667	0.295
Lhcb1	T30	13.2		
Lhcb1	T30	13.07		
Lhcb1	C50	22.85	22.81	0.115326
Lhcb1	C50	22.68		
Lhcb1	C50	22.9		
Lhcb1	T50	21.58	22.95	0.125033
Lhcb1	T50	22.95		
Lhcb1	T50	24.32		

Table 8.23: The PCR efficiency of Lhcb

			cycle #
	cDNA input		crossing threshold
dilution	**cDNAamount**	lncDNA	**Ct (or CP)**
1x	1	0	8.98
3x	0.333333333	-1.09861229	9.22
9x	0.111111111	-2.19722458	14.98
linear regr. of Ct / ln(DNA):		k	-2.730718
		d	8.060000
		y = kx + d	
slope (calc. for 10x dil.)			-6.2877
PCR efficiency			**1.4422**

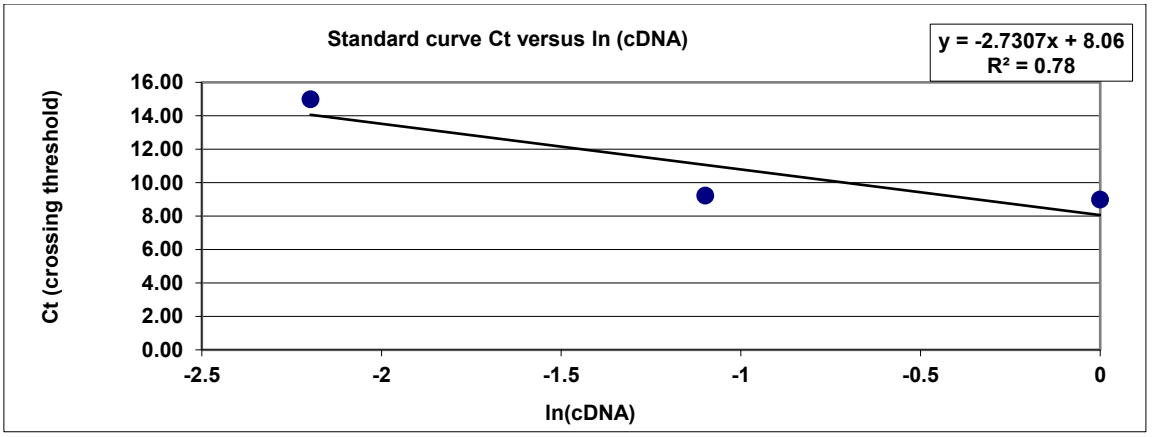


Figure 8.34: Standard curve versus in (cDNA) for Lhcb

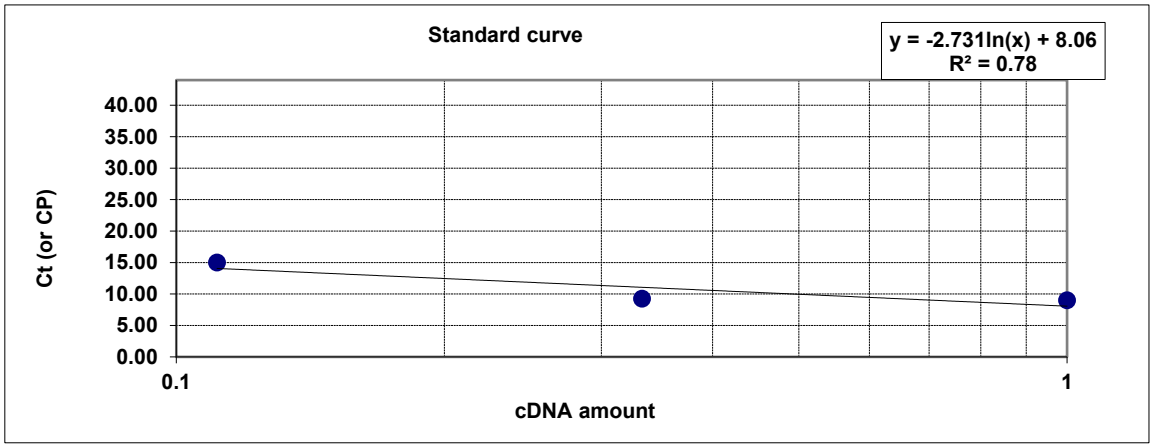


Figure 8.35: Standard curve of Lhcb

Table 8.24: The fold of induction of Lhcb

	Target gene	Reference gene	
PCR efficiency	1.4422	1.1267	
Ct values	**Ct target**	**Ct reference**	**fold induction**
Control	22.81	6.61	1
1mg	9.505	19.01333	464.2488339
5mg	8.1733	19.55	373.3316479
10mg	10.85	7.58	238.6923553
20mg	13.19687	7.33	142.2729236
30mg	22.95	7.475	37.45950453
50mg	10.08	7.215	1.021101206

Table 8.25: Mean Cq and Fold Induction of LhcbGene.

Sample	Gene	Mean Cq	Fold of Induction
C	Lhcb1	22.81	1
1 mg	Lhcb1	10.08	464.248834
5 mg	Lhcb1	10.85	373.331648
10 mg	Lhcb1	8.1733	238.692355
20 mg	Lhcb1	9.505	142.272924
30 mg	Lhcb1	13.19667	37.4595045
50 mg	Lhcb1	22.95	1.02110121

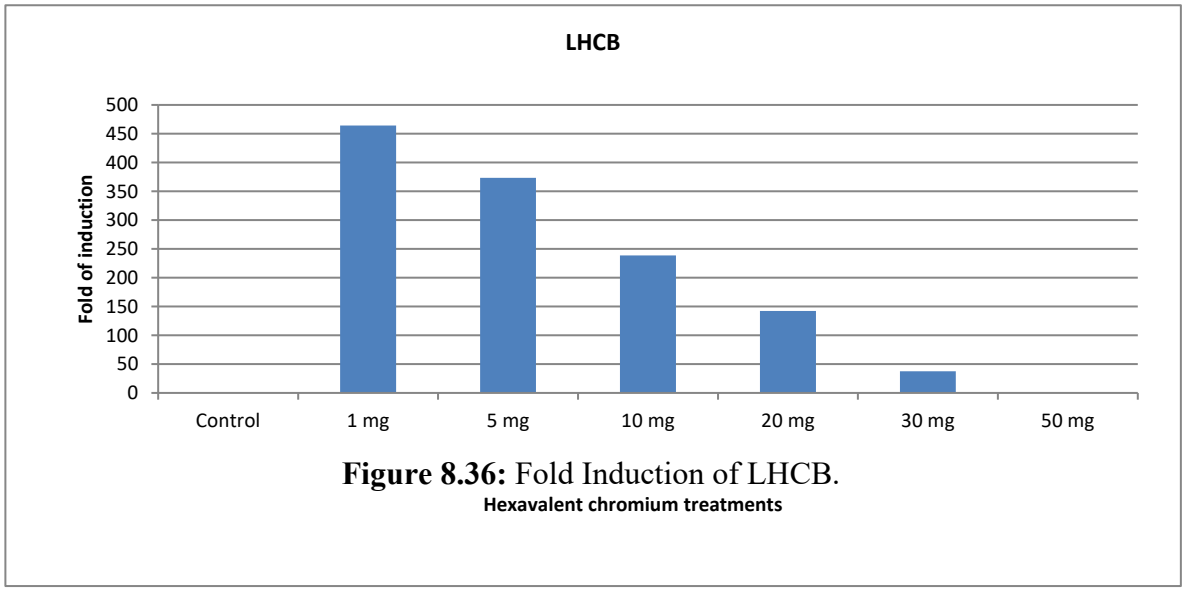


Figure 8.36: Fold Induction of LHCB.

GSTU21

Glutathione S-transferases (GSTs) are a large complex family of enzymes that protect the cells from oxidative damage arising due to heavy metal stress. In the studies conducted by qRT PCR, GSTtau21 increases gradually and reaches a maximum at 10 mg chromium treated with a fold of induction of 154.64 and decreases with further increase in concentration upto 50 mg chromium stress with an expression of 62.78 reveals a very efficient antioxidant system getting disrupted slowly due to heavy metal Cr^{VI} stress (table 8.29 and figure 8.39).

Table 8.26: CQ values of GSTU21

Gene Name	Tube Name	CQ value	Mean CQ	Std. deviation
GSTU21	C1	24.65	24.645	0.00707
GSTU21	C1	24.65		
GSTU21	C1	24.64		
GSTU21	T1	18.97	19.0333	0.08505
GSTU21	T1	19		
GSTU21	T1	19.13		
GSTU21	C5	24.65	24.645	0.00707
GSTU21	C5	24.65		
GSTU21	C5	24.64		
GSTU21	T5	14.86	14.99	0.13
GSTU21	T5	14.99		
GSTU21	T5	15.12		
GSTU21	C10	24.65	24.645	0.00707
GSTU21	C10	24.65		
GSTU21	C10	24.64		
GSTU21	T10	16.04	15.92	0.12
GSTU21	T10	15.92		
GSTU21	T10	15.8		
GSTU21	C20	24.65	24.645	0.00707
GSTU21	C20	24.65		
GSTU21	C20	24.64		
GSTU21	T20	13.31	17.1408	0.39
GSTU21	T20	12.92		
GSTU21	T20	12.53		
GSTU21	C30	24.65	24.645	0.00707
GSTU21	C30	24.65		
GSTU21	C30	24.64		
GSTU21	T30	13.76	13.92	0.16
GSTU21	T30	13.92		
GSTU21	T30	14.08		
GSTU21	C50	24.65	24.645	0.00707
GSTU21	C50	24.65		
GSTU21	C50	24.64		
GSTU21	T50	14.39	14.5767	0.18502
GSTU21	T50	14.58		
GSTU21	T50	14.76		

Table 8.27: The PCR efficiency of GSTU21

			cycle #
	cDNA input		crossing threshold
dilution	**cDNA amount**	lncDNA	**Ct (or CP)**
1x	1	0	12.20
3x	0.333333333	-1.09861229	16.12
9x	0.111111111	-2.19722458	17.68
		k	-2.494055
		d	12.593333
linear regr. of Ct / ln(DNA):		y = kx + d	
slope (calc. for 10x dil.)			-5.7428
PCR efficiency			**1.4932**

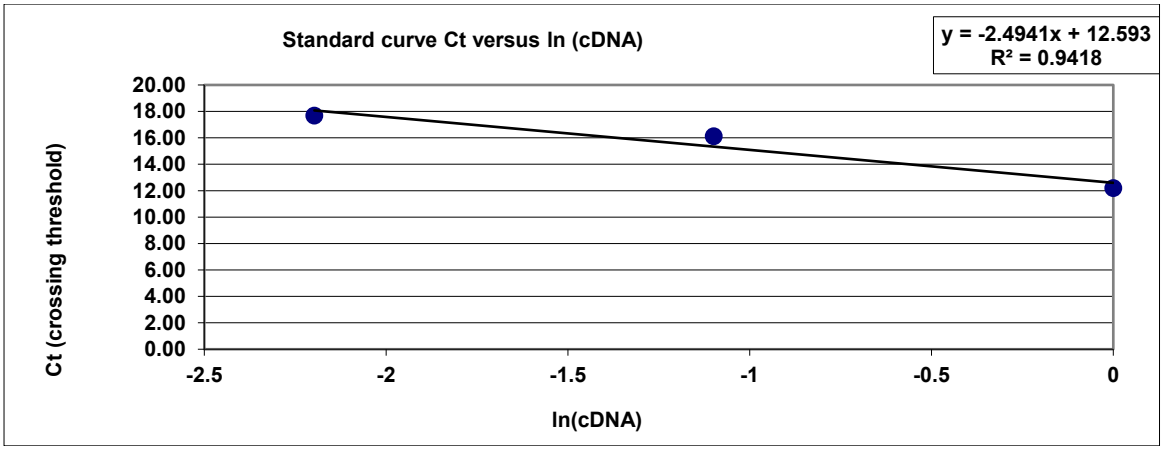


Figure 8.37: Standard curve versus in (cDNA) for GSTU21

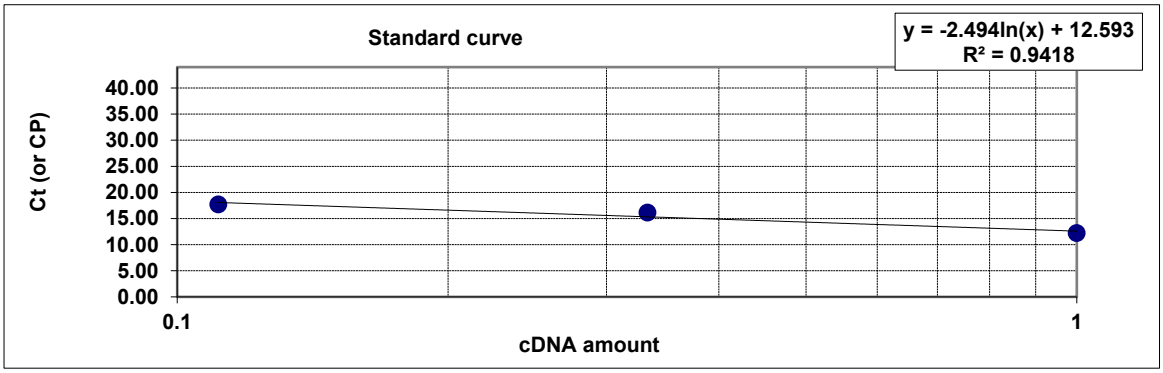


Figure 8.38: Standard curve for GSTU21

Table 8.28: The fold of induction GSTU21

	target gene	reference gene	
PCR efficiency	1.4932	1.1267	
Ct values	**Ct target**	**Ct reference**	**fold induction**
control	24.645	6.61	1
1mg	19.0333	7.215	10.19600954
5mg	14.99	7.58	53.8696879
10mg	15.92	19.55	154.6426626
20mg	17.14077	19.01333	88.9155454
30mg	13.92	7.33	80.29776255
50mg	14.57667	7.475	62.78773222

Table 8.29: Mean Cq and Fold Induction of GSTU21 Gene.

Sample	Gene	Mean Cq	Fold of Induction
C	GSTU21	24.645	1
1 mg	GSTU21	19.0333	10.19600954
5 mg	GSTU21	14.99	53.8696879
10 mg	GSTU21	15.92	154.6426626
20 mg	GSTU21	17.14077	88.9155454
30 mg	GSTU21	13.92	80.29776255
50 mg	GSTU21	14.57667	62.78773222

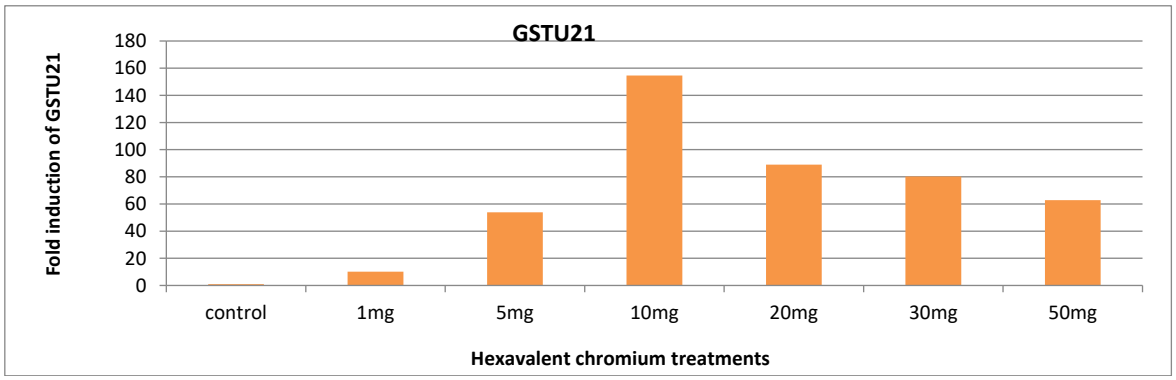


Figure 8.39: Fold Induction of GSTU21

CONCLUSION

The present study provides an insight into the partial molecular mechanisms underlying Cr stress in *Amaranthus dubius* through transcriptome sequencing and RT qPCR analysis. Gene regulation of ABC transporters, metal ion binding, antioxidant systems, transcription factors, stress related genes and photosystems implies the disruption of homeostasis due to effect of hexavalent chromium (Cr^{VI}) stress and all these implies that Cr^{VI} induced oxidative toxicity has profound damaging effect on the leafy vegetable *A. dubius*. The down-regulated mode in the majority gene expressions of biological, cellular and molecular functions and the trend in the fold induction of selected stress responsive genes at higher Cr^{VI} stress levels clearly substantiate the disturbances and alterations in various biological growth processes and related inhibition in morphological growth, biomass and quality recorded in *A. dubius* plants. The finding of stress responsive genes may provide the scope for the breeding of stress tolerant varieties of Amaranth in the future.

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CHAPTER-9

GENERAL DISCUSSION

Now a day, one of the most common and significant issue encountered by agriculturists in many countries is the environmental pollution arising from heavy metals. Uncontrolled increment in the accumulation of heavy metals in water, air and land is the most menacing environmental threat owing to rapid industrialization. The effluents and waste water from industries are likely to carry both organic and inorganic components including excess levels of heavy metals. The shortage of clean water, especially in urban areas, has resulted in the use of such polluted industrial waste water for irrigating food crops, mainly leafy vegetables and fruits (Arora *et al.,* 2008). There are misconceptions among the farmers that these effluents are rich in nutrients promoting quick vegetative growth and thereby help them to reduce fertilization expense. This is one of the major factors that tempt the farmers to promote the use of industrial wastes (Swaminathan & Vaidheeswaran, 1991; Ahmad *et al.,* 2006), unaware of the detrimental consequences of its chemical compounds on the anatomy, physiology, genetical and cytological characteristics and hence the morphology, growth and productivity as well as the quality of the crops (Wyszkowski & Wyszkowska, 2003; Kovacic & Nikolic, 2005). The various observations on the ill effects of heavy metal contaminants indicate that when the concentration exceeds a certain level, these pollutants are toxic to plants. Therefore, the environmental pollution due to heavy metal is a matter of serious concern as it is a threat to the food security in the future and further, the vegetables we intake are usually strong accumulators of heavy metals which pose a high health risk. In human, about 90% of the total heavy metal intake has been reported to be through the consumption of vegetables and fruits while it is only 10% through dermal contacts and inhalation of metal contaminated dust (Martorell *et al*. 2011; Khan *et al*. 2014). Hence it is a fact that heavy metal pollution is a risk factor directly or indirectly for the primary consumers like humans and animals (Zeller & Feller, 1999).

Among various heavy metals, the accumulation of chromium (Cr) and particularly the hexavalent chromium (Cr^{VI}) in agricultural soils is a global concern at present. This is due their higher demand in several industrial applications and corresponding heavy discharge of chromium-containing effluents leading to their significant contamination in the environment (Joutey *et al*., 2015). Till now, any essential biological role of metal Cr^{VI} in plant growth and development have not been reported, while at the same time, it is generally perceived that their excessive levels in plant tissues induce severe toxic effects. This toxic effect is mostly correlated with the stress induced by the generation of excessive reactive oxygen species (ROS) which cause impairment in the normal biological characteristics of plants. However, in contrast to the seriousness that have been given to the impact of toxic metals such as cadmium, mercury and lead, the impact of Cr toxicity has not yet received much attention from scientists and researchers although the bioavailability of hexavalent chromium in the agricultural land is increasing at an alarming rate and that may possibly limit production and quality of crops particularly with respect to the vegetable crop species, which are a major dietary source rich in essential nutrients consumed all over the world. It is in this contest that the present study has been carried out in *Amaranthus dubius* Mart. ex Thell., an easily cultivable and widely consumed leafy vegetable crop species (CO-1) of Amaranthus which is rich in minerals, vitamins and essential micronutrients, and is often referred to as “Poor man’s vegetable”.

The present investigation on the impact of different levels of hexavalent chromium (Cr^{VI}) stress on morphological growth parameters, biomass yield and quality of *Amaranthus dubius* undoubtedly revealed that the heavy metal Cr^{VI} is toxic and has significant inhibitory effects. The majority of the parameters such as length of shoot, root and total plant, fresh and dry biomass of shoot, root and total plant and similarly, the total number of leaves and total leaf area per plant recorded a progressive reduction in their values over the control in progression with the increase in Cr^{VI} stress, with certain exceptions in the initial Cr^{VI} stress of 1mg Cr^{VI} with respect to root length, which recorded an increase over control. The R/S ratio in terms of fresh weight and dry weight and similarly the DW/FW ratio in terms of shoot, root and total plant generally tend to exhibit progressive

decrease over control up to 20-30mg Cr^{VI} stress, while further increase in the stress caused a reversal and showed a gradually increasing trend in the ratios up to the highest Cr^{VI} stress of 70mg selected for the study. With an exception of R/S ratio obtained at 70mg Cr^{VI} stress, all these increases did not exceed over the ratios obtained in control plants. The morphological growth responses obtained to the increasing concentration of metal Cr^{VI} indicate a progressive increasing trend in the inhibition of plant growth (IPG) and a corresponding reduction in tolerance index (TI). The inferences in this aspect revealed that the intensity of Cr^{VI} stress is negatively correlated with TI and positively correlated with IPG and this in turn indicate dose dependent Cr^{VI} toxicity on growth performance. The restricted absorption and translocation of water and mineral nutrients due to reduced root growth, root injury and impaired root penetration which have led to imbalance and alterations in the normal physiology and metabolism and further, genome instability, disruption in structural organization and integrity of cell, disturbed cell division and enlargement etc. induced under Cr^{VI} stress and toxicity might have additively contributed to the significant reduction in overall growth performance of *A. dubius* (Breckle, 1989; Samantaray *et al*., 1998; Zou *et al*., 2006; Srivastava & Jain, 2011; Saddiqe *et al*., 2015). Therefore, it is highly relevant to evaluate these processes under Cr^{VI} stress to scientifically prove and substantiate that toxicity hindered growth, productivity and quality.

The differences observed and recorded on various physiological and biochemical parameters between the Cr^{VI} treated and control plants of *A. dubius* in the investigation clearly indicate that Cr^{VI} stress and its toxicity is capable of causing disruption and alterations in physio-chemical process. The availability of heavy metals in toxic level in the growing medium is reported to cause disruption in many vital activities in plants. The metal toxicity on electron carriers and the flow rate of electron from water to PS-II reaction center and similarly, inhibitory action on ATP production and energy level of cells have been reported (Prasad *et al*., 1991). The disruption in the biosynthesis of chlorophyll molecules and activities of many relevant enzymes by interfering with the functional role of certain essential metals which are acting as cofactors or stimulating agent in many physiological and metabolic processes is reported (Hossain, 2012; Ali,

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to a medium level Cr^{VI} stress treatment could be the result of increased transcript levels of the genes encoding them as part of mitigating the harmful effects of free radicals. However, the lipid peroxidation and cell damage induced by severe Cr^{VI} toxicity at higher stress levels might have hindered the transcript levels of those genes and reduce their defense mechanism against the ROS, as reflected by the drastic reduction in growth and biomass (Omosun *et al*., 2008). Therefore, it is certain that the disturbances and alterations to various physiological and biochemical process induced by Cr^{VI} stress could have taken part in the reduction of morphological growth parameters of *A. dubius*.

The mineral nutrients are very essential for various physiological and metabolic pathways in plants for the normal growth and development. However, the stress due to excess concentration of heavy metals in the growing environment is known to disturb the uptake and translocation of mineral nutrients in plants due to different reasons. The results of the present study inferred that the excess concentration of non-essential Cr^{VI} stress in the growing medium affected the uptake of both macro and micro nutrient elements and their accumulation in the shoot component of *A. dubius*. The results revealed considerable and progressive inhibition in the uptake of macro elements like N, P, K, Ca and Mg along with the progressive increase in the Cr^{VI} stress, with certain exceptions at initial level Cr^{VI} stress of 1mg with respect to K and Ca, where an insignificant increase in the uptake over the control is noticed. However, the uptake of micronutrients exhibits a different trend compared to macronutrients where the content of Fe, Zn and Mn in the shoot component progressively increased over the control up to Cr^{VI} stress of 20-30mg and then onwards, further increase in the stress caused decrease in their content, in which the decreases in Zn content are still maintained higher over the control. The increase in the content of micro elements like Fe, Zn & Mn and macro elements like K & Ca recorded during the lower level Cr^{VI} stress may be as part of the mechanism of plant to impart tolerance to Cr^{VI} toxicity by enhancing the activity of these nutrient dependent antioxidant enzymes. With respect to the uptake and accumulation of metal Cr, gradual and progressive increase has been recorded up to the highest stress of 70mg Cr^{VI} and revealed a dose dependent increase in their content. The reduced root surface area and impaired root penetration as a result of metal toxicity induced damage and poor root system development could be the major reason that contributed towards the significant

reduction in uptake and accumulation of majority of the macro and micro elements at higher Cr^{VI} stress (Dahiya *et al*., 2003). Further, it can be attributed due to the masking of sorption sites and forming insoluble complexes by the metal Cr^{VI} (Osu & Onyema, 2016) or by the displacement of certain elements from physiological binding sites by the metal Cr^{VI} having structural similarity (Mengel & Kirkby, 2018). The investigation in general revealed that there exists of a positive correlation between essential mineral element uptake with physio-chemical growth process as well as morphological growth performances and all these parameters are in turn negatively correlated with intensity of Cr^{VI} stress and toxicity.

The oxidative stress under excessive concentration of heavy metal in the growing environment has an adverse impact on the plant anatomy leading to undesirable structural and ultrastructural changes and damages in cells followed by retarded growth process and biomass production as reported by researchers (Zhang *et al.,* 2009; Rahoui *et al.,* 2010; Balaji *et al*., 2011). The data obtained from anatomical parameters in the present study also inferred similar trend which indicate that excessive concentration of heavy metal Cr^{VI} is toxic to *A. dubius* and is capable of causing disturbances and damages to the internal structures affecting the structural integrity and biomass production. It was Shanker *et al*. in 2005 who inferred from his study that disruption in the structural integrity and deformation in the quantitative characteristics of vascular elements in both root and stem of *Cicer arietinum* is caused in response to undesirable level of heavy metal stress. A study conducted on *Vigna* species using heavy metals like cadmium and chromium reported the disorganization of epidermis, distortion of cortical cell and stelar region in stem and root (Ratheesh *et al*., 2010). Similarly, a study performed in the brake fern plants by Balaji *et al.* (2011) reported cellular breakdown and crumbling of internal structural elements in the roots and leaves when exposed to heavy metal stress. The toxic impacts of hexavalent chromium on the anatomical parameters of *A. dubius* observed in the present study includes disturbed shape and arrangement of cells, reduced cell number and size, waviness in the cell wall, cell damages and deformation in the epidermal, cortical and stelar regions of root, stem and leaf and gradual loss of overall structural integrity. All these toxic impacts on anatomical parameters inferred in the study could be due to excess generation of reactive oxygen species which beyond the control limits of

various antioxidant activities leads to oxidative impairment at cellular level. The observations and inferences obtained in the present study are in support of and in agreement with majority of the previous said reports. Further, most of these disturbances in the anatomy and corresponding decrement in growth and biomass production are found to be gradually increasing with the enhancement in the Cr^{VI} stress and revealed that the intensity of Cr^{VI} stress is negatively correlated with the internal structural integrity and biomass production.

The green leafy vegetables are rich in vitamins, amino acids, fatty acids, dietary fibre etc. and are indispensable for good health and proper functioning of immune system, which can be made available only through dietary food intake as it cannot be synthesized in our body. However, the enhancing industrialization and related environmental contamination in the recent years have affected the nutritional quality of crop plants. The oxidative damage induced by heavy metal stress can cause chloroplast degradation and peroxidation of lipids, which in turn can cause a negative effect on the nutritional quality of crop plants. The results of nutritional quality analysis carried out in *Amaranthus dubius* revealed that the content of majority of the nutritional components like Vit K, Vit B3, Vit B6, Vit B9, amino acid lysine and dietary fiber generally showed a decreasing trend with increasing Cr^{VI} stress, with the exception of low-level Cr^{VI} stress of 1mg for Vit B6 and fatty acid content and 1mg & 5mg Cr^{VI} for Vit B9, which remained unchanged over the control. The decreasing trend in the content of these nutritional components with increasing Cr^{VI} stress might be due to their utilization in various activities as part of tolerating oxidative stress. This may include utilization of vitamin K for reducing the intensity of injury due to the depletion of glutathione (Li *et al*., 2003), Vitamin B6 for scavenging activity of free radicals (Higashi, 2006), vitamin B3 for reducing cell damage (Lanska, 2010), vitamin B9 for protecting cells against lipid peroxidation (Gliszczynska, 2007) etc. Therefore, the study clearly indicates the existence of a negative correlation between the content of these nutritional components and the intensity of Cr^{VI} stress in *A. dubius* and these inferences are in similar lines with the decreasing vitamin content and other nutritional components against increasing concentration of heavy metal reported by Pleasants *et al*. (1992), Munzuroglu *et al*. (2005), Gill and Saggoo (2010) and Widowati (2012). However, the content of Vit A

showed an increasing trend up to 20mg Cr^{VI} stress and then starts to decrease progressively at further higher stress levels. The increase in the content of Vit A and the maintenance of Vit B3 and fatty acids (C18:0, C18:1, and C18:2) content at relatively lower level Cr^{VI} stress recorded in the study might be due to their increased production by the plant to take part in antioxidant activity to reduce oxidative damage. This inference in the study is supported by the report of Le Guédard *et al*. (2008), Lanska (2010), Malivindi *et al*. (2018) and Shimizu *et al*. (2018) that nutrients like Vit A, Vit B3 and fatty acids have some radical scavenging activities directly or indirectly in plants against stresses and capable of bringing down the level of oxidative stress. The drastic and significant reduction in the content of most of the selected nutritional parameters inferred in the study, particularly at higher level Cr^{VI} stress of 50mg & 70mg revealed toxicity induced oxidative damage and reduction in overall nutritional value of the crop.

The analysis of chromosomal behavioural changes and mitotic index in the root tip cell of *Amaranthus dubius* under Cr^{VI} stress indicates that Cr^{VI} is genotoxic and cytotoxic. The chromosomal aberrations at anaphase and metaphase stage such as misorientation, clumping, stickiness, fragmentation and pulverization are the major abnormalities generally recorded at comparatively lower-level stress of 1mg - 10/20mg Cr^{VI}. The maximum diverse types of chromosomal aberrations are detected and recorded at10mg Cr^{VI} stress. The interference of metal Cr^{VI} in the mitotic phase of cell cycle through the generation of reactive oxygen species (ROS) causing disturbances in the spindle formation leading to disruption in the orientation of chromosome might be the major reason for various chromosomal abnormalities recorded in the study (Selim *et al*., 1981; Kwankua *et.al*., 2012; Singh, 2015). Further increase in the Cr^{VI} stress in the study seems to cause drastic reduction in chromosomal aberrations and are mostly restricted to stickiness of chromosomes. The disturbances in the folding of chromosome fibres that link the chromatids by sub-chromatid bridges (McGill *et al*., 1974) together with the damage caused to the proteins which form an integral part of the chromosomes (EI-Sadek, 1972) may have a major impact on chromosome at this stage of Cr^{VI} stress leading to stickiness of chromosomes. However, at the same time, it is the stage at which the non-chromosomal abnormalities such as prophase disturbances like disturbed spindle, condensed and fragmented chromatids and the interphase disturbances like nuclear

lesion, binucleate cells, nuclear bulging and elongation, micronuclei, cells without nucleus and misplaced nucleus start to be detected. The frequency in the detection of prophase disturbances increased progressively up to 30mg Cr^{VI} stress and then start to decrease at 50mg and 70mg Cr^{VI} stress while the frequency of interphase disturbances increased drastically and progressively up to the highest Cr^{VI} stress of 70mg. The higher frequency of cells having interphase disturbances at higher level Cr^{VI} stresses indicate cytotoxic effects. The study further noticed a progressive decrease in number of dividing cells and corresponding reduction in mitotic index as the Cr^{VI} stress is increasing. This clearly indicate dose dependent mitodepressive activity of Cr^{VI} stress and this can be attributed to the disruption in the G1 stage of cell cycle affecting DNA synthesis and nuclear proteins (Mohandas & Grant, 1972; Yekeen *et al*., 2011). The overall observations in the study revealed the intensity of Cr^{VI} stress is negatively correlated to mitotic index whereas it is positively correlated to induction of overall abnormality percent and these inferences in the study are in similar lines with the observations of Abu Ngozi (2012) and Singh (2015) in *Allium cepa* L. under heavy metal contamination. The impaired gene expression and related protein production due to disrupted genome stability and chromosomal behaviour induced by the activity of reactive oxygen species (ROS) under Cr^{VI} stress could be one of the major reasons for the physiological and biochemical disorders inferred in the study which in turn might have contributed to the inhibition in morphological growth, biomass production and quality. These observations are in agreement with Dutta *et al*. (2018) who reported genome instability and DNA damage due to ROS induced oxidative stress leading to depleted biosynthesis as well as damage to many macro and micro molecules like proteins, enzymes, lipids, amino acids, vitamins etc. of physiological and metabolic functions for the normal growth and developments of plants.

The molecular mechanism behind the disruptions and alterations in the biological growth characteristics of *Amaranthus dubius* under Cr^{VI} stress has not been analyzed before. The comparative analysis of RNA sequences of plants treated with highest stress of 70mg Cr^{VI} and the control plants using the FASTQ quality information analysis revealed 13.78Gb data expression for control plants and 19.1Gb data expression for the Cr^{VI}

stressed plants. The differential gene expression analysis performed using Cuffdiff program showed 790 genes in which 307 genes are up-regulated and 483 genes are down-regulated under Cr^{VI} stress. The analysis of the result indicates the majority of the genes expressions of biological, cellular process and molecular functions are in a down-regulated mode when compared to that of up-regulated. The data further revealed that the genes coding for several stress related proteins like phytochelations, heat shock proteins, antioxidant enzymes; proteins involved in transmembrane transport and vacuolar transport and certain transcription factors are seen up-regulated. On the contrary, the genes coding for many transcription factors like WRKY DNA binding proteins, B box type Zinc family proteins are seen down-regulated. The quantification using RT qPCR of 4 selected stress responsive candidate genes of interests SOD, CAT, Glutathione-S-Transferases (GSTU21) and Lhcb1Ah1 from the differentially expressed genes revealed the fold induction is low in comparatively lower stress levels of 1mg and 5mg Cr^{VI} and then increased to their highest fold induction of 4078.11, 11.75 and 464.25 in 20mg Cr^{VI} stressed *A. dubius* plants respectively for SOD, CAT and Lhcb1Ah1 genes while the highest fold induction of 154.64 for GSTU21 gene has been recorded at 10mg Cr^{VI} stressed plants. Further increases in the Cr^{VI} stress caused decline in fold induction for all the selected genes. The down-regulated mode in the majority gene expressions of biological, cellular and molecular functions and the trend in the fold induction of selected stress responsive genes revealed through transcriptome profiling and RT qPCR analysis clearly substantiate the disturbances and alterations in various biological growth processes and related inhibition in morphological growth, biomass and quality obtained in *A. dubius* plants under hexavalent chromium (Cr^{VI}) toxicity. The down-regulated mode in the gene expressions of biological, cellular and molecular functions and similarly, reduction in the fold induction of selected stress responsive genes at higher Cr^{VI} stress levels could be due to the disrupted transcript levels of these genes under Cr^{VI} toxicity (Omosun *et al*., 2008; Saffar *et al*., 2009).

The present investigation clearly revealed that the hexavalent chromium (Cr^{VI}) is inhibitory in action and toxic to *Amaranthus dubius*. The Cr^{VI} metal has no positive role in normal growth and development of *A. dubius* and instead, their presence in excessive concentration in the growing environment caused significant reduction in morphological

growth, productivity and nutritional quality. The inference obtained in the study further indicates dose dependent Cr^{VI} toxicity and clearly revealed the intensity of Cr^{VI} stress positively correlated with inhibition of plant growth (IPG%) while negatively correlated with tolerance index (TI%) as well as the nutritional quality of *A. dubius* plant. The oxidative toxicity induced by the generation of excessive reactive oxygen species (ROS) under Cr^{VI} stress have caused alterations and impairment in most of the biological characteristics of the plant. The Cr^{VI} toxicity induced disruption and inhibition in physiological and biochemical process, reduction and imbalances in mineral elements and water uptake, chromosomal and non-chromosomal abnormalities and reduction in mitotic index due to genome instability and cytotoxicity, loss of structural integrity, reduced cell growth and damage are all negatively and additively impacted to the significant reduction in overall morphological growth parameters, productivity and nutritional quality of *A. dubius*. The study noted that even though the overall productivity of *A. dubius* in terms of fresh and dry biomass are more or less reduced in all the Cr^{VI} treated plants over the control, the slight improvements recorded in certain parameters of *A. dubius* plants treated with lowest-stress level of 1mg Cr^{VI} over the control and that might be as part of mitigating role by the plant to tide over the stress at relatively lower level. The study concludes that the hexavalent chromium (Cr^{VI}) stress induced oxidative damage caused significant reduction in productivity and nutritional quality of *A. dubius* and similarly, it also exhibits a progressive and dose dependent increase in the accumulation of toxic metal chromium, which poses a threat to the human health. Further, the study suggests the alterations and the correlation of various physiological, biochemical, anatomical, genetical and cytological characteristics with corresponding morphological growth responses observed and recorded in the investigation due to hexavalent chromium (Cr^{VI}) stress may be useful indicators to identify Cr^{VI} stress in *A. dubius* in an area in the future.

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CHAPTER-10

SUMMARY AND CONCLUSION

The major parts of the agricultural land around the world are contaminated with heavy metals due to the population explosion-oriented impact of enormous increase in industrialization and geochemical activities. Now a day, among various heavy metals, the bioavailability of hexavalent chromium (Cr^{VI}) in the agricultural land is increasing at an alarming rate due to their higher demand in several industrial applications followed by heavy discharge of chromium-containing effluents into the open environment. The metal Cr^{VI} does not have any known biological role in plant growth but it is generally perceived that their excessive levels in the growing environment can induce toxic effects which negatively impact the growth performance of crops plants particularly the leafy vegetable crop species which are the major dietary source rich in essential nutrients. *Amaranthus dubius* Mart. ex Thell., is such kind of a widely consumed, easily cultivable leafy vegetable which is best suited for tropical humid regions. The present study evaluated the impact of different levels of hexavalent chromium (Cr^{VI}) stress on the growth performance and quality of *Amaranthus dubius* (CO-1).

Healthy uniform sized potted seedlings of *A. dubius* in triplicate blocks of 30 each are selected and treated with different specific Cr^{VI} stress of 1mg, 5mg, 10mg, 20mg, 30mg, 50mg & 70mg Cr^{VI}/kg potting mixture. 0.0mg Cr^{VI}/kg potting mixture is taken as control. The analysis on the morphological growth and biomass production, toxicity level in terms of tolerance index (TI in %) and inhibition of plant growth (IPG in %), physiological and biochemical parameters, uptake of macro and micro elements and metal Cr, anatomical parameters, geno-cytotoxic effects, nutritional quality and Transcriptome profiling and RT qPCR have been carried out.

The analysis of morphological growth parameters of *Amaranthus dubius* plants in general revealed that the metal Cr^{VI} is toxic and has significant inhibitory effect on shoot, root and total plant length, fresh and dry biomass of shoot, root and total plant and the number of leaves and total leaf area per plant over the control plant. The R/S ratio and DW/FW ratio of Cr^{VI} stressed plants generally tend to exhibit progressive reduction over control

up to 20-30mg Cr^{VI} stress and then turn to show a gradual increasing trend on further increase in Cr^{VI} stress. The analysis of morphological growth and biomass production clearly revealed that the tolerance index (TI%) is negatively correlated while the inhibition of plant growth is positively correlated with the intensity of Cr^{VI} stress and this in turn indicates dose dependent Cr^{VI} toxicity.

In order to confirm and substantiate the hindered morphological growth and significant reduction in productivity, the study analysed and evaluated various biological growth process and characteristics of *A. dubius* plants to reveal different factors that have contributed to the inhibition in overall growth performance compared to the control plants. The results obtained from the analysis of various physiological and biochemical processes revealed disruptions and alterations caused by Cr^{VI} stress. The parameters such as chlorophyll b, total chlorophyll, stomatal conductance (g_S), intercellular CO_2 concentration (Ci), transpiration rate (E), net photosynthetic rate (P_N), content of starch, total carbohydrate, content of fat and protein generally recorded a progressive decrease in their values parallel with increase in the Cr^{VI} stress and clearly revealed the existence of a positive correlation of these parameters with morphological growth and biomass production. The mineral elements are very essential for various physiological and metabolic pathways in plants for the normal growth and development. The analysis of mineral elements in the shoot component under Cr^{VI} stress in the study indicates a dose dependent inhibition in the uptake and accumulation of macro (N, P, K, Ca and Mg) and microelements (Fe, Zn and Mn), with certain exceptions in lower levels of Cr^{VI} stress, whereas with respect to the metal Cr, it revealed a dose dependent gradual progressive increase in its content in the shoot component. In the light of the knowledge that the oxidative stress induced by excess heavy metals in the growing environment has an adverse effect on the plant anatomy, the present study made an attempt to analyse the impact of Cr^{VI} stress on the anatomical parameters and growth in *A. dubius*. The data obtained indicates Cr^{VI} stress has caused reduction in cell size & number, irregularity in cell shape and arrangements, sinuosity in the cell wall, collapse of structural integrity and cell damages in root, stem and leaves, reduction in number & size of stomata and stomatal index. The inferences in the study clearly indicate Cr^{VI} stress has significant

toxic impact on anatomy which is capable of causing disturbances and damages to the internal structures with a negative impact on biomass productivity. The inhibition of root growth is the most predominant and one of the first morphological change usually observed in plants growing under heavy metal stress. This may be because the root is the part which is in direct contact with the soil environment and susceptible to the oxidative stress affecting the genome stability, gene expression and mitotic activity in root cells. In this respect the present study also analysed mitotic index and chromosomal behavioural changes in the root tip cell of *A. dubius*. The results of the study indicated that the Cr^{VI} stress is genotoxic and cytotoxic. At comparatively lower-level stress of 1mg - 10/20mg Cr^{VI}, diverse types of chromosomal aberrations at anaphase and metaphase stages are found dominating. Further increase in the Cr^{VI} stress seems to cause drastic reduction in frequency and diversity of chromosomal aberrations and are mostly restricted to stickiness of chromosomes. However, at the same time the non- chromosomal abnormalities related to prophase and interphase disturbances were detected to begin from 10/20mg Cr^{VI} stress onwards and the frequency of non- chromosomal abnormalities increased drastically and progressively towards higher level Cr^{VI} stress. The study further noticed a progressive decrease in number of dividing cells and corresponding reduction in mitotic index as the Cr^{VI} stress is increasing and clearly indicate dose dependent mitodepressive activity. The overall inference revealed that the intensity of Cr^{VI} stress is negatively correlated with mitotic index whereas it is positively correlated with the induction of overall abnormality percent. The nutritional quality of any crop is known to be influenced by the physical, chemical and biological environment in which it grows. The present study also analysed the nutritional quality of *A. dubius* plant to reveal whether the Cr^{VI} toxicity has any deteriorating effect or not. The results revealed that the content of majority of the nutritional components like Vit K, Vit B3, Vit B6, Vit B9, Vit A and the content of fatty acids, amino acid lysine and dietary fiber generally showed a decreasing trend with increasing Cr^{VI} stress, with certain exceptions at low-level stress. The drastic and significant reduction in the content of most of the selected nutritional parameters particularly at higher level Cr^{VI} stress of 50mg & 70mg revealed toxicity induced oxidative damage and related reduction in overall nutritional quality of the crop.

This inference clearly indicates the existence of a negative correlation between the nutritional quality of the crop and the intensity of Cr^{VI} stress.

The inhibitions and alterations observed in most of the biological growth process and characteristics of *Amaranthus dubius* plants exposed to hexavalent chromium (Cr^{VI}) stress are agreeable and correlated with their reduced morphological growth performance, retarded biomass yield and reduced nutritional quality over the control plants. In order to further confirm and substantiate all these inferences and correlations, the study carried out molecular level analysis in terms of gene expression. The most reliable approach to get a better insight into the differential gene expression pattern is Transcriptome profiling and RT qPCR analysis. To get an understanding of Cr^{VI} stress induced differential gene expression patterns, a comparative analysis of RNA sequence between *A. dubius* plants treated with the highest Cr^{VI} stress of 70mg and the control has been carried out. From the differentially expressed genes, 4 stress responsive candidate genes SOD, CAT, GSTU21 and Lhcb1Ah1 have been selected for quantification using RT qPCR among plants treated with 1mg to 50mg Cr^{VI} stress and control. The functional classification of differentially expressed genes (DEGs) are performed using Genome Ontology (GO) annotation system and the DEGs are categorized based on their biological, cellular and molecular functions. All the RNA sequence reads are deposited at NCBI site with bio-accession number SUB11418745. The FASTQ quality information analysis revealed 13.78Gb data expression for control plants and 19.1Gb data expression for the Cr^{VI} stressed plants. The differential gene expression analysis performed using Cuffdiff program showed 790 genes in which 307 genes are up-regulated and 483 genes are down-regulated under Cr^{VI} stress. The down-regulated mode in the majority gene expressions of biological, cellular and molecular functions and the Low-Up-Down trend in the fold induction of selected stress responsive genes in accordance with low-Medium-High Cr^{VI} stress levels clearly substantiate the inhibitions and alterations in various biological growth process and related significant reductions in morphology, biomass yield and nutritional quality of *A. dubius* plants.

Therefore, the present investigation clearly reveal that the hexavalent chromium (Cr^{VI}) is inhibitory in action and toxic to *Amaranthus dubius* and the intensity of toxicity is dose dependent. The Cr^{VI} toxicity induced impairment and inhibition in physiological and biochemical process, reduction and imbalances in the uptake of mineral elements and water uptake, chromosomal and non-chromosomal abnormalities and reduction in mitotic index due to genome instability and cytotoxicity, loss of structural integrity, reduced cell growth and damage all together negatively and additively impacted the significant reduction in overall morphological growth performance, productivity and nutritional quality of the plant. The study further noted certain exceptions like an increase in the content of nutrient components Vit A, Vit B3 and fatty acids and mineral elements like K, Ca, Fe, Zn and Mn at lower levels of Cr^{VI} stress. These nutrient components as well as mineral elements are reported to have some antioxidant role directly or indirectly in plants against stresses. Such increases at relatively lower level Cr^{VI} stress may be due to an attempt by the plant to take part in the mitigation process by way of radical scavenging activity to reduce oxidative damage. The study put forth that the metal toxicity, in general, hampers the yield and quality of *Amaranthus dubius* and likewise other crop plants significantly which may lead to a severe threat in food security in future. Further, the accumulation of heavy metals by vegetable crop plants may pose a serious threat to human health on consumption, which is a matter of great concern.

APPENDIX - I

List of the selected genes of interest from the DEGs deposited at the NCBI Sequence Read Archive (SRA) in response to Cr^{VI} Stress in *Amaranthus dubius*

Gene ID	log2(fold_change)	Regulation	arabi-defline
AH016052	-5.3	Down	17.6 kDa class II heat shock protein
AH021830	-3.3	Down	A20/AN1-like zinc finger family protein
AH001941	-2.4	Down	Actin-like ATPase superfamily protein
AH000371	3.72	Up	arogenate dehydratase 6
AH017446	2.69	Up	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein
AH017567	4.6	Down	atypical CYS HIS rich thioredoxin 5
AH007839	3.63	Up	auxin response factor 8
AH006815	2.31	Up	basic helix loop helix (bHLH) DNA binding superfamily protein
AH006815	2.31	Up	basic helix loop helix (bHLH) DNA binding superfamily protein
AH010546	3.08	Up	basic helix loop helix (bHLH) DNA binding superfamily protein
AH013370	2.6	Down	basic helix loop helix (bHLH) DNA binding superfamily protein
AH017378	-2.7	Down	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AH020057	-2.3	Down	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AH019857	-4	Down	basic region/leucine zipper motif 53
AH016718	-6.9	Down	branched-chain amino acid transaminase 2
AH001229	-4.4	Down	C2H2-type zinc finger family protein
AH020504	-4.9	Down	calmodulin (CAM)-binding protein of 25 kDa
AH006716	2.44	Up	calmodulin binding
AH014213	-2.3	Down	calmodulin-binding family protein
AH021595	3.11	Up	Calmodulin-binding protein
AH002455	3.5	Up	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein
AH019052	4.6	Down	cationic amino acid transporter 2
AH002741	4.95	Up	chlorophyll A/B binding protein 1
AH019182	4.3	Down	CHY type/CTCHY type/RING type Zinc finger protein
AH022740	-3.3	Down	CONSTANS-like 2
AH012808	-2.3	Down	copper-exporting ATPase / responsive-to-antagonist 1 / copper-transporting ATPase (RAN1)
AH012808	-2.3	Down	copper exporting ATPase / responsive to antagonist 1 / copper transporting ATPase (RAN1)
AH003232	2.84	Up	cytochrome P450, family 706, subfamily A, polypeptide 4
AH003233	4.51	Up	cytochrome P450, family 706, subfamily A, polypeptide 4
AH018776	-3.6	Down	cytochrome P450, family 707, subfamily A, polypeptide 1
AH007929	2.81	Up	cytochrome P450, family 77, subfamily A, polypeptide 4
AH015074	-2.4	Down	cytochrome P450, family 81, subfamily D, polypeptide 3
AH021397	2.61	Up	cytochrome P450, family 82, subfamily C, polypeptide 4
AH016607	4.06	Up	cytochrome P450, family 83, subfamily B, polypeptide 1
AH017656	3.66	Up	cytochrome P450, family 86, subfamily A, polypeptide 2
AH012400	-2.8	Down	disease resistance family protein / LRR family protein
AH015249	-4.4	Down	erf domain protein 9
AH009885	-3.5	Down	ethylene responsive element binding factor 4
AH010083	-2.3	Down	ethylene responsive element binding factor 4
AH021674	4.3	Down	ethylene responsive element binding factor 4
AH009885	3.5	Down	ethylene responsive element binding factor 4
AH010083	2.3	Down	ethylene responsive element binding factor 4
AH021674	4.3	Down	ethylene responsive element binding factor 4
AH011400	3.4	Down	ethylene responsive element binding factor 5
AH018319	2.8	Down	ethylene responsive element binding factor 5
AH011400	3.4	Down	ethylene responsive element binding factor 5
AH018319	2.8	Down	ethylene responsive element binding factor 5
AH010709	2.64	Up	expansin A13
AH016417	4.01	Up	expansin A16
AH013694	3.33	Up	expansin A6
AH000340	3.38	Up	expansin B3
AH002341	-2.7	Down	expansin-like A2
AH000758	-4.4	Down	expansin-like A3
AH016732	-2.4	Down	FKBP-type peptidyl-prolyl cis-trans isomerase family protein
AH003899	-3.3	Down	Gibberellin-regulated family protein
AH001022	3.29	Up	glutathione S-transferase TAU 21
AH001024	3.75	Up	glutathione S-transferase TAU 21
AH018636	-2.1	Down	glutathione S-transferase THETA 1

AH021504	2.15	Up	heat shock protein 70 (Hsp 70) family protein
AH014784	-2.6	Down	heat shock transcription factor A4A
AH019542	2.79	Up	heat shock transcription factor B2A
AH008650	3.69	Up	heavy metal atpase 2
AH008650	3.69	Up	heavy metal atpase 2
AH015481	2.25	Up	Heavy metal transport/detoxification superfamily protein
AH018596	-3.4	Down	Heavy metal transport/detoxification superfamily protein
AH012098	-2.6	Down	Heavy metal transport/detoxification superfamily protein
AH011922	-2.6	Down	homeobox 7
AH001248	2.24	Up	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein
AH001248	2.24	Up	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein
AH008028	-3.3	Down	homogentisate 1,2-dioxygenase
AH010676	-3.3	Down	HSP20-like chaperones superfamily protein
AH013668	-2.9	Down	HSP20-like chaperones superfamily protein
AH023024	-3	Down	HSP20-like chaperones superfamily protein
AH013392	2.72	Up	jasmonate-zim-domain protein 1
AH013392	2.72	Up	jasmonate-zim-domain protein 1
AH001301	-3.9	Down	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AH008152	-4.4	Down	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AH001647	2.51	Up	Leucine-rich repeat (LRR) family protein
AH020345	-3.2	Down	Leucine-rich repeat (LRR) family protein
AH012266	2.57	Up	LRR and NB-ARC domains-containing disease resistance protein
AH011351	2.23	Up	Major facilitator superfamily protein
AH002029	-4.2	Down	MATE efflux family protein
AH002029	-4.2	Down	MATE efflux family protein
AH012349	-4.5	Down	MATE efflux family protein
AH015007	-2.8	Down	MATE efflux family protein
AH001294	-2.2	Down	mitogen-activated protein kinase 3
AH022649	-3.2	Down	mitogen-activated protein kinase kinase kinase 13
AH013895	-5.6	Down	mitogen-activated protein kinase kinase kinase 13
AH002006	2.54	Up	multidrug resistance-associated protein 3
AH017339	3.18	Up	multidrug resistance-associated protein 3
AH017339	3.18	Up	multidrug resistance-associated protein 3
AH012928	3.35	Up	multidrug resistance-associated protein 4
AH012928	3.35	Up	multidrug resistance-associated protein 4
AH018382	-3.4	Down	NA
AH000666	2.28	Up	NA
AH022599	3.35	Up	NA
AH008092	-3.5	Down	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein
AH010413	-2.7	Down	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein
AH008092	-3.5	Down	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein
AH010413	-2.7	Down	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein
AH017564	-3	Down	NAC domain containing protein 100
AH017564	-3	Down	NAC domain containing protein 100
AH005222	-2.1	Down	NAC domain containing protein 83
AH005222	-2.1	Down	NAC domain containing protein 83
AH005032	-3.8	Down	NAC-like, activated by AP3/PI
AH005032	-3.8	Down	NAC-like, activated by AP3/PI
AH021250	-2.5	Down	N-MYC downregulated-like 1
AH006841	2.54	Up	Pathogenesis-related thaumatin superfamily protein
AH001424	-2.9	Down	Peroxidase superfamily protein
AH003820	-3.4	Down	Peroxidase superfamily protein
AH006244	-2.7	Down	Peroxidase superfamily protein
AH013236	3.79	Up	Peroxidase superfamily protein
AH021501	2.5	Up	Peroxidase superfamily protein
AH021976	-3	Down	Peroxidase superfamily protein
AH016040	-2.3	Down	peroxin 10
AH004988	-6.1	Down	phosphoenolpyruvate carboxykinase 1
AH008008	2.94	Up	phosphoenolpyruvate carboxylase 3

AH008672	2.75	Up	Phosphoenolpyruvate carboxylase family protein
AH007883	2.92	Up	phosphoenolpyruvate carboxylase kinase 1
AH000855	3.59	Up	proline rich protein 4
AH021126	5.44	Up	RING/U box superfamily protein
AH001703	-3.2	Down	SAUR-like auxin-responsive protein family
AH001716	-3.2	Down	SAUR-like auxin-responsive protein family
AH010367	-3.4	Down	SAUR-like auxin-responsive protein family
AH010368	-3.9	Down	SAUR-like auxin-responsive protein family
AH022329	-2.5	Down	SAUR-like auxin-responsive protein family
AH005259	2.42	Up	SUPPRESSOR OF AUXIN RESISTANCE1
AH004348	2.99	Up	Thioredoxin superfamily protein
AH010202	-3.8	Down	Thioredoxin superfamily protein
AH012862	-2.8	Down	Thioredoxin superfamily protein
AH015267	-4.3	Down	Thioredoxin superfamily protein
AH023020	-3.6	Down	UDP-Glycosyltransferase superfamily protein
AH009055	2.1	Down	Vacuolar ATPase assembly integral membrane protein VMA21-like domain
AH018671	4.1	Down	winged helix DNA binding transcription factor family protein
AH000655	-3.6	Down	WRKY DNA-binding protein 40
AH000655	-3.6	Down	WRKY DNA-binding protein 40
AH014651	-4.3	Down	xylem NAC domain 1
AH014651	-4.3	Down	xylem NAC domain 1
AH020558	-4.4	Down	xyloglucan endotransglucosylase/hydrolase 15
AH020562	-2.9	Down	xyloglucan endotransglucosylase/hydrolase 24
AH011552	-2.1	Down	xyloglucan endotransglucosylase/hydrolase 30
AH018452	-3.4	Down	xyloglucan endotransglucosylase/hydrolase 30
AH008757	-5	Down	Xyloglucan endotransglucosylase/hydrolase family protein
AH015743	-8	Down	Xyloglucan endotransglucosylase/hydrolase family protein
AH008758	-6.7	Down	xyloglucan endotransglycosylase 6
AH004768	-5.8	Down	xyloglucan:xyloglucosyl transferase 33

APPENDIX - II

LIST OF PUBLICATIONS AND ABSTRACTS

A. Research Papers in peer reviewed International/ National

- Simi, R., & Dr.Subin, M.P. (2015).Phytotoxic effect of Hexavalent chromium on Total Protein Fatty acid and Dietary Fibre Content of *Amaranthus dubius* Mart. ex Thell. *PARIPEX-Indian Journal of Research*, 4 (12), 35-37.

- Simi, R., & Dr.Subin, M.P. (2022). Hexavalent Chromium $Cr^{(VI)}$ Uptake Induced Physiological Effects and Growth in *Amaranthus dubius* Mart. ex Thell. *Applied Ecology and Environmental Sciences*, 10(1), 1-10.

B. Research papers in seminar/ conference/ book paper presentation

- Simi R & Dr.Subin M.P (2018). Genotoxic and Cytotoxic effects of Hexavalent Chromium (Cr^{VI}) Stress in *Amaranthus dubius* Mart. ex Thell. International Conference on Phytomedicine from 29 th to 31 st August 2018. Organised by Dept of Botany, Bharathiar University, Coimbatore.

- Simi.R. & Subin. M. P. (2020). The Impact of Chromium (Cr^{VI}) Stress on the Structural Integrity, Antioxidant Enzyme Activity and Growth in *Amaranthus dubius* Mart. ex Thell. International Conference on Climate change: Adaptation and Mitigation, From 8th to 10th January 2020, organized by Finishing School Cell, St. Thomas College (Autonomous), Thrissur, Kerala. Paper Presented and published in Proceedings of International Conference on Climate change: Adaptation and Mitigation (ISBN: 978-81-944730-0-8; Pages-130-134).

Research Paper Botany

Phytotoxic Effect of Hexavalent Chromium on Total Protein, Fatty Acid and Dietary Fibre Content of Amaranthus Dubius Mart.ex Thell

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ABSTRACT

Hexavalent chromium (CrVI) is a serious environmental pollutant and contamination of soil and water by this metal is a matter of serious discussion. Toxic effects of Cr on plant growth and development which affect the total dry matter production and yield is due to its deleterious effects on plant physiology and metabolism. The present study was aimed at assessing the phytotoxic effect of hexavalent chromium on Amaranthus dubius Mart. Ex Thell raised over standard potting mixture which is amended with different concentration levels of hexavalent chromium. Potassium dichromate salt (K2Cr2O7) was used as the source of chromium. The parameters considered and analyzed for the study includes fatty acid, total protein and total dietary fibre content. The investigation reveals fatty acid and total dietary fibre content of A. dubius is having a negative correlation with concentration of metal chromium in the potting mixture. However, the overall protein content exhibited a positive correlation during lower level concentration treatments whereas negative correlation was observed at higher levels

KEYWORDS Amaranthus dubius Mart. Ex Thell, hexavalent chromium stress, fatty acid, total protein and total dietary fibre

INTRODUCTION

Chromium is a toxic and nonessential element to plants and hence they do not possess specific mechanisms for its uptake. However, plants uptake this heavy metal through carriers used for the uptake of essential metals for plant metabolism. Soil and ground water contamination due to heavy metal chromium resulting from various anthropogenic activities has become a serious source of concern to plant and animal scientists over the past decade. The impact of Cr toxicity on the physiology and metabolism of plants depends on the metal speciation, which determines its uptake, translocation, accumulation and resultant toxicity in the plant system. Hexavalent chromium (Cr^{VI}) is considered the most toxic form of Cr as it is a strong oxidant with a high redox potential in the range of 1.33–1.38 eV accounting for a rapid and high generation of ROS and its resultant toxicity (Shanker et al., 2004a, b). Hexavalent chromium compounds are used in industries for metal plating, leathering, tanning, wood preservation etc. and these anthropogenic activities have led to the widespread contamination and have increased its bioavailability and biomobility (Arun et al., 2005). However, in contrast to other toxic trace metals like mercury, lead, cadmium etc, Cr has not received much attention from plant scientists. The aim of the present study was to investigate the effects of hexavalent chromium on the content of fatty acid, total protein and total dietary fibre in ***Amaranthus dubius*** Mart. Ex Thell.

MATERIALS AND METHODS

Healthy seeds of ***Amaranthus dubius*** collected from Kerala Agricultural University, Thrissur is subjected to germination and healthy germinated seeds which emerge out of the growing media are transferred carefully into plastic pots containing standard potting mixture prepared by using farmyard manure, red soil and sand in the ratio 1:1:1. One week after transplantation, healthy uniform sized and aged seedlings are selected in triplicate blocks of 30 each for each treatment. Treatments include eight different specific concentration levels of heavy metal chromium which are selected based on a preliminary germination study. Different concentrations include 1mg Cr/kg potting mixture, 5mg Cr/kg potting mixture, 10mg Cr/kg potting mixture, 20mgCr/kg potting mixture, 30mg Cr/kg potting mixture, 50mg Cr/kg potting mixture, 70mg Cr/kg potting mixture and 0.00mg Cr/kg potting mixture (control). Different concentrations of metal chromium are prepared from potassium dichromate salt ($K_2Cr_2O_7$) of an analytical reagent grade. The required concentrations of chromium are applied to the potting mixture as aqueous solution in such a way that every 200ml contains required quantities of chromium. 200ml of distilled water alone is used as control (0.0mg Cr/200ml). Plant materials for analysis were collected from tender shoot parts of all treated plants on twentieth day of metal treatment. Different parameters such as fatty acid content, total protein content and total dietary fibre content were selected and analyzed for all the Cr treated as well as the control plants of ***A. dubius,*** to determine the impact of different levels of Cr^{VI} stress.

Estimation of Fatty Acids Content

Fatty acid content was estimated by the method of Cox and Pearson (1962). Dissolve 1 to 10g of sample in 50ml of the neutral solvent in a 250 ml conical flask. Add a few drops of 1% phenolphthalein. Titrate the content against 0.1N potassium hydroxide. Shake constantly until a pink color, which persists for fifteen seconds, is obtained.

Calculation

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titer value x Normality of KOH x 56.1}}{\text{Weight of the sample (g)}}$$

Fatty acid is calculated as oleic acid using the equation 1ml N/10KOH =0.028g oleic acid.

Estimation of Total Protein Content

Protein was estimated by the method of Lowry et al. (1951). 100 mg plant material was homogenized with 3 ml of 10% trichloroacetic acid. The homogenate was centrifuged at 10,000 rpm and supernatant was discarded. The pellets obtained after centrifugation were treated with 3 ml 1 N NaOH, heated on water bath for 7 minutes and cooled down to room temperature. Again, the solution was centrifuged for five to ten minutes at 5000 rpm. 5 ml reagent containing 100 parts of 2% solution of sodium carbonate and one part of 2% solution of sodium potassium tartrate was added to

0.5 ml of supernatant thus obtained after centrifugation and allowed to stand for ten to fifteen minutes. Then 5 ml Folin and Ciocalteu's Phenol reagent (diluted with distilled water in ratio of 1:1) was added and allowed to stand for half an hour for development of colour, and then finally absorbance was measured at 700 nm.

Estimation of Total Dietary Fibre Content

Total dietary fibre (TDF) content was estimated by the method followed by Prosky et al. (1988). With each assay, run blanks along with samples to measure any contribution from reagents to residue. Weigh out duplicate 1.0g samples accurately into 400 ml tall-form beakers. Add 40 ml MES-TRIS blend buffer solution (pH 8.2) to each beaker. Stir on magnetic stirrer until sample is completely dispersed in solution. Incubation with heat-stable α-amylase: Add 50 µl heat-stable α-amylase solution, while stirring at low speed. Cover each beaker with aluminium foil and incubate for 30 min with continuous agitation in a shaking water bath at 98-100°C. Remove all sample beakers from shaking water bath and cool to 60°C. Scrape any ring around beaker and gels in bottom of beaker with spatula and rinse side wall of beaker and spatula with 10 ml distilled water by using pipette. Adjust temperature of water bath to 60°C by draining some of hot water from water bath and adding cold water. Add 100 µl protease solution to each sample. Cover with aluminium foil and incubate for 30 min with continuous agitation in a shaking water bath at 60±1°C. Remove sample beakers from shaking water bath. Dispense 5 ml of 0.56 1N HCl solution into sample while stirring. Check pH and adjust to 4.1-4.8 by adding either 5% NaOH solution or 5% HCl solution. Add 200 µl amyloglucosidase solution while stirring on magnetic stirrer. Cover with aluminium foil and incubate for 30 min with continuous agitation in a shaking water bath at 60±1°C. To each sample, add 225 ml 95% EtOH pre-heated to 60°C. Ratio of EtOH volume to sample volume should be 4:1. Cover all samples with aluminium foil and incubate at room temperature for 60 min to allow precipitate to form. Tare crucible containing Celite to nearest 0.1 mg. Wet and redistribute the bed of Celite in the crucible, using 15 ml of 78% EtOH from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as an even mat. Quantitatively transfer all the precipitate and suspension from each beaker by using a wash bottle with 78% EtOH and a rubber spatula, to its respective crucible. Use vacuum and wash the residue successively with two 15 ml portions of (a) 78% EtOH, (b) 95% EtOH and (c) Acetone. Dry the crucible containing residue overnight in an air oven at 103°C. Cool crucible in desiccators for approximately 1 h. Weigh crucible containing dietary fibre residue and Celite to nearest 0.1 mg. To obtain residue weight, subtract tare weight, i.e. weight of dried crucible and Celite. Protein and ash determination: One residue sample and the corresponding blank are analyzed for protein using Kjeldahl method. Use 6.25 factors for all cases to calculate gram of protein. For ash analysis, incinerate the second residue sample and corresponding blank for 5 hours at 525°C. Cool in desiccators and weigh to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash content.

Calculations:

$$\text{Dietary Fibre (\%)} = \frac{\frac{R1+R2}{2} - p - A - B}{\frac{m1+m2}{2}} \times 100$$

Where:

R1 = residue weight 1 from m1; R2 = residue weight 2 from m2; m1 = sample weight 1; m2 = sample weight 2; p = protein weight from R_1; A = ash weight from R_2 and B = blank

$$B = \frac{BR_1 + BR_2}{2} - BP - BA$$

Where:

BR = blank residue; BP = blank protein from BR1 and BA = blank ash from BR2.

RESULT AND DISCUSSION

Effect of different concentrations of hexavalent chromium (Cr^{VI}) on the content of fatty acid, total protein and total dietary fibre in ***Amaranthus dubius*** Mart. Ex Thell is shown in table 1. Fatty acid content showed a decreasing trend with progressive increase in Cr concentration. Reduction in fatty acid content was more prominent from a concentration of 20 mg Cr treatment per kg potting mixture onwards. Maximum reduction was recorded at the highest concentration of 70mg Cr/ kg potting mixture while the least reduction was recorded at 5mg Cr/ kg potting mixture and was 34.2% and 2.1% respectively over control; however the treatment of 1mg Cr did not contribute to any change in concentration of fatty acid content (Figure 1). The decrease in fatty acid content in ***A.dubius*** observed in the present study when subjected to increasing concentration of heavy metal Cr may be attributed to the decomposition of polyunsaturated fatty acids under oxidative stress. This may be considered as one of the potential parameter due to heavy metals toxicity. The present inferences are in similar lines with the observations of Le Guédard et al. (2008) in ***Lactuca sativa*** and Fikriye (2013) in ***phaseolus vulgaris*** seedlings which are exposed to different concentration of heavy metals including chromium, showed a decrease in fatty acid composition linearly with increased heavy metal concentration.

TABLE 1
Effects of different concentrations of hexavalent chromium (Cr^{VI}) on the content of fatty acid, total protein and total dietary fibre in *Amaranthus dubius*

Treatment Cr^{VI} (mg/kg soil)	Fatty acid content calculated as oleic acid (g)	Total Protein content (mg/g)	Total dietary fibre content (%)
1mg	0.187±0.012	18.07±0.12	43±0.82
5mg	0.183±0.012	18.2***±0.14***	42.67±0.47
10mg	0.180±0.014	17.8±0.16	41.67±0.94
20mg	0.160±0.009	17.13±0.48	34±3.27
30mg	0.140±0.016	11.4±1.39	28.33±3.40
50gm	0.130±0.163	6.56±0.94	26.33±2.62
70mg	0.123±0.012	5.9±0.50	26± 1.63
Control	0.187±0.005	18.03***±0.12***	43.33±0.47

The data presented in the table 1 indicates an increasing trend in total protein content with lower level concentrations and decreasing trend with higher level concentrations of hexavalent chromium (Cr^{VI}) in ***A. dubius***. Slight increase in the overall protein content recorded in 1mg Cr/ kg potting mixture and 5mg Cr/kg potting mixture was 0.22% and 0.94% respectively over control. Stress due to lower level concentrations of heavy metal has been reported to induce a variety of proteins including various enzymes and proline, resulting in an overall increase in protein content (Shah and Dubey. 1997). However the study reveals further increase in the concentration of metal Cr found to cause reduction in total protein content and maximum reduction was recorded at treatment of 70mg Cr/kg potting mixture and was 67.28% reduction over control (Figure 2). Decrease in total protein content in ***A.dubius*** observed at higher level concentrations may be because of enhanced protein degradation process under increased protease or other catabolic enzyme activities together with induced lipid peroxidation and fragmentation of proteins due to increased Cr toxicity. Decrease in the synthesis of proteins due to disturbance in nitrogen metabolism under Cr toxicity may also be the reason contributed (Auda and Ali, 2010).

Figure 3 and table 1 summarize the results for the effects of different concentrations of hexavalent chromium (Cr^{VI}) on total dietary fibre content of ***A.dubius.*** Data reveals a negative correlation exist between total dietary fibre content and concentration of heavy metal Cr. The present investigation observed

the maximum TDF content was obtained in lowest concentration of 1mg Cr/kg potting mixture (43±0.82%) which was 0.76% decrease over control and further decreases as the concentration of Cr increases. The decrease was more prominent from treatment of 20mg Cr/kg potting mixture onwards and the lowest TDF content was recorded in plants treated with highest concentration of 70mg Cr/kg potting mixture and it was about 40% decrease over control. The present study results are in conformity with earlier findings of Gill and Saggoo (2010) in turnip plants and Emmanuel et al. (2013) in eight different edible vegetable plants, when subjected to chromium stress. Decrease in plant growth due to low utilization of nitrogen together with reduction in photosynthesis and degradation of important cellular components under oxidative stress resulting from Cr toxicity might be the reason for reduction in TDF content.

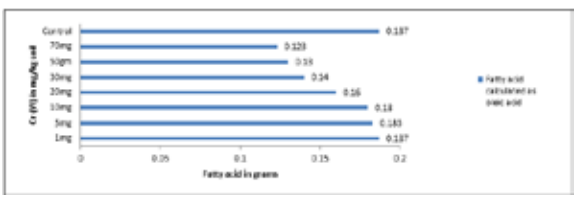

Figure1: Changes in fatty acid content of *Amaranthus dubius* under different concentrations of Cr^{VI}. Data are mean of 3 replicates

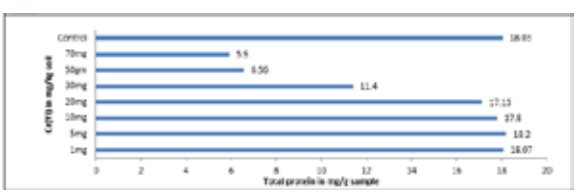

Figure 2: Changes in total protein content of *Amaranthus dubius* under different concentrations of Cr^{VI}. Data are mean of 3 replicates

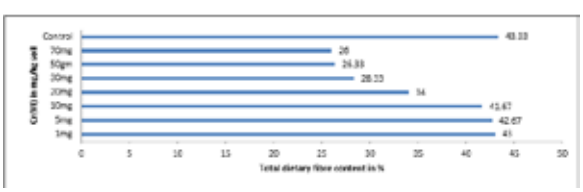

Figure 3: Changes in total dietary fibre content of *Amaranthus dubius* under different concentrations of Cr^{VI}. Data are mean of 3 replicates

Figure 4: Difference in growth performance of *Amaranthus dubius* at 20th day after treatment with different concentrations of hexavalent chromium (Cr^{VI})

CONCLUSION

In the present study, exposure to hexavalent chromium (Cr^{VI}) affected different parameters of ***Amaranthus dubius*** Mart. Ex Thell: fatty acid content, overall protein content and total dietary fibre content. Exposure of ***A. dubius*** seedlings to Cr^{VI} decreased all the parameters selected for the study with an exception during the low level concentration treatment with respect to overall protein content which might be due to the induction of certain proteins including enzymes and proline by the plant as an attempt to resist the oxidative stress due to chromium toxicity. The decreasing trend was found to increase as the concentration of the metal chromium is increasing in the growing media and hence the investigation clearly reveals, Cr^{VI} is toxic to ***A. dubius.*** The degree of toxicity is concentration dependant and as the concentration of metal chromium is increasing, the quality as well as the yield of the plant is severely affected.

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Hexavalent Chromium (Cr^{VI}) Uptake Induced Physiological Effects and Growth in *Amaranthus dubius* Mart. ex Thell

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Abstract The heavy metal chromium (Cr) is considered as one of the most hazardous metal ion which hampers crop productivity by contaminating both soil and water due to higher solubility. The present study evaluated the impact of different levels of hexavalent chromium (Cr^{VI}) stress on the morphological and physiological growth performance of *Amaranthus dubius.* The morphological growth parameters such as length of shoot, root and total plant, fresh and dry biomass accumulation, root/shoot ratio and total leaf number and leaf area/plant recorded progressive reduction along with the progressive increase in the Cr^{VI} stress, with certain exceptions with respect to the length of root and total plant at initial level Cr^{VI} stress and root/shoot ratio at higher level concentrations. With respect to physiological parameters, 1mg Cr/kg soil treatment caused a stimulatory action on stomatal conductance (g_S)**,** intercellular CO_2 concentration (C_i) and net photosynthetic rate (P_N) while further enhancement in the concentration of Cr^{VI} is inhibitory in action. The Cr^{VI} toxicity at higher level stresses of 50mg Cr/kg soil and above has significant deleterious effects and the tolerance index is reduced to 6.1 - 2.9% and inhibition of plant growth enhanced to 93.9 - 97.1% in terms of biomass yield compared to control plants.

Keywords: *Amaranthus dubius, Cr^{VI} toxicity, physiological and morphological growth*

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1. Introduction

The accelerated rate of industrialization and unscientific disposal of industrial effluence leading to heavy metal pollution is a major environmental problem. This situation is alarming in developing countries where untreated heavy metal containing wastewater from industries is extensively used for irrigation or is disposed off carelessly into open environments [1]. These metals are deposited in different soil profiles leading to long-term metal contamination. Metals are mostly absorbed by plants easily and prove toxic, which can be observed as deterioration in quality and retardation of growth and productivity [2]. Heavy metal chromium is a nonessential element and at higher concentrations they are toxic to plants. Hexavalent chromium (Cr^{VI}) is considered more toxic form which is a strong oxidant with a high redox potential accounting for a faster and over generation of reactive oxygen species (ROS) which disturb redox balance, affecting the water relations and physiology and hence inhibits growth performance in plants [3]. The requirement of Cr^{VI} compounds in diverse industries for metal plating, leathering, tanning, wood preservation etc. have led to widespread contamination. Even though, their increased bioavailability in the agricultural land is actually a major concern regarding the productivity and quality of crop plants, in contrast to toxic metals such as cadmium, mercury and lead, the impact of Cr toxicity has not received much attention from scientists and researchers. The leafy vegetables represent an excellent component of the habitual diet in the tropical countries. It is known that leafy vegetables accumulate higher amounts of heavy metals in leaves than in roots, stems and fruits [4]. As the metal pollutants are non-degradable and are readily taken up by crop plants, these are likely to enter easily into the food chain causing serious health hazards to human beings [5]. Amaranth is one of the easily cultivable leafy vegetable which requires less attention and expense and also it is the cheapest leafy vegetable available in the market although it is rich in minerals, vitamins and essential micronutrients and is often referred to as "Poor man's vegetable". The present study is carried out to evaluate the impact of Cr^{VI} stress and toxicity on morphology, physiology and related growth performance and biomass yield of *Amaranthus dubius* Mart. ex Thell., a cultivable crop species (CO1) of Amaranthus which is best suited for tropical humid regions. It is an erect, annual herb growing up to an average of 90cm tall. The leaves are simple having ovate to rhomboid-ovate shaped lamina arranged spirally around the slender stem.

2. Materials and Methods

Healthy seeds of *Amaranthus dubius* after germination are transferred to plastic pots containing standard potting mixture prepared by using farmyard manure, red soil and sand in the ratio 1:1:1. One week after transplantation, healthy uniform sized seedlings are selected in triplicate blocks of 30 each for each treatment. Seven different concentration levels of hexavalent chromium (Cr^{VI}) are prepared from potassium dichromate salt (K2Cr2O7) of analytical reagent grade and are 1mg, 5mg, 10mg, 20mg, 30mg, 50mg and 70mg Cr^{VI}/kg soil. The required concentrations of Cr^{VI} are applied to the potting mixture as aqueous solution in such a way that every 200ml contains required quantities of Cr^{VI}. 200ml of distilled water alone is used as control. The evaluation of physiological growth parameters are carried out on the 25th day of Cr^{VI} treatments while the morphological growth parameters are carried out on the 45th day of Cr^{VI} treatments.

2.1. Morphological Growth Parameters

- Length of shoot, root and total plant
- Total number of leaves and total leaf area/plant
  Leaf area measurements are done using leaf area meter (Model LI - 3100C, LI-COR Biosciences, USA.
- Fresh and dry biomass
- Root/Shoot fresh and dry weight ratio
- The tolerance index (TI) [6]
  TI (%) = 100 x (Fresh biomass in metal treatment)/ (Fresh biomass in control)
- Inhibition of plant growth (IPG) [7]

$$\text{IPG}\ (\%) = \frac{\left(\begin{array}{l}\text{Total biomass of control}\\ -\text{Total biomass in metal treatment}\end{array}\right)}{\text{Total biomass in control}} \text{X100}$$

2.2. Physiological Growth Parameters

- Net photosynthetic rate (P_N)
- Stomatal conductance (g_S)
- Intercellular CO_2 concentration (Ci)

The study analyses the physiological parameters related to photosynthesis by using the LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA). The device is fitted with blue and red LEDs (LI-6400-02B) as a light source for the relevant photosynthetic measurements. All the leaf gas exchange properties were recorded under the sample chamber condition where photosynthetic photon flux density (PPFD) was at 1000 $\mu Mm^{-2}s^{-1}$, leaf temperature was at 25°C, and the CO_2 level was at 380μM CO_2 mol^{-1}. The leaf gas exchange measurements are measured on the second fully expanded leaf from the top of the plant during the period at 9:00 am –11:00 am. The data presented on each parameter is an average of six readings taken from six plants from each treatment.

2.3. Statistical Analysis of Data

Statistical analysis of data is carried out using Duncan's Multiple Range Test of SPSS statistics 20.

3. Results and Discussions

3.1. Length of Shoot, Root and Total Plant

The effect of different concentrations of hexavalent chromium (Cr^{VI}) on the length of shoot, root and total plant of *Amaranthus dubius* is depicted in the Figure 1. Cr^{VI} concentration treatments caused progressive reduction in the length of shoot, root and total plant over the control plants along with the increase in the concentration of treatments, with an exception in the case of 1mg Cr^{VI}/kg soil treatment with respect to root and total plant length, where an enhancement of 14.55% and 4.22% occurred for root and total plant length respectively over the control. The highest reduction of 74.66% for shoot length, 86.42% for root length and 78.83% for total plant length is obtained with 70mg Cr^{VI} treatment while the lowest reduction of 2.53% is obtained with 1mg Cr^{VI} treatment for shoot length whereas, 68.09% and 51.36% respectively for root and total plant length obtained with 5mg Cr^{VI}.

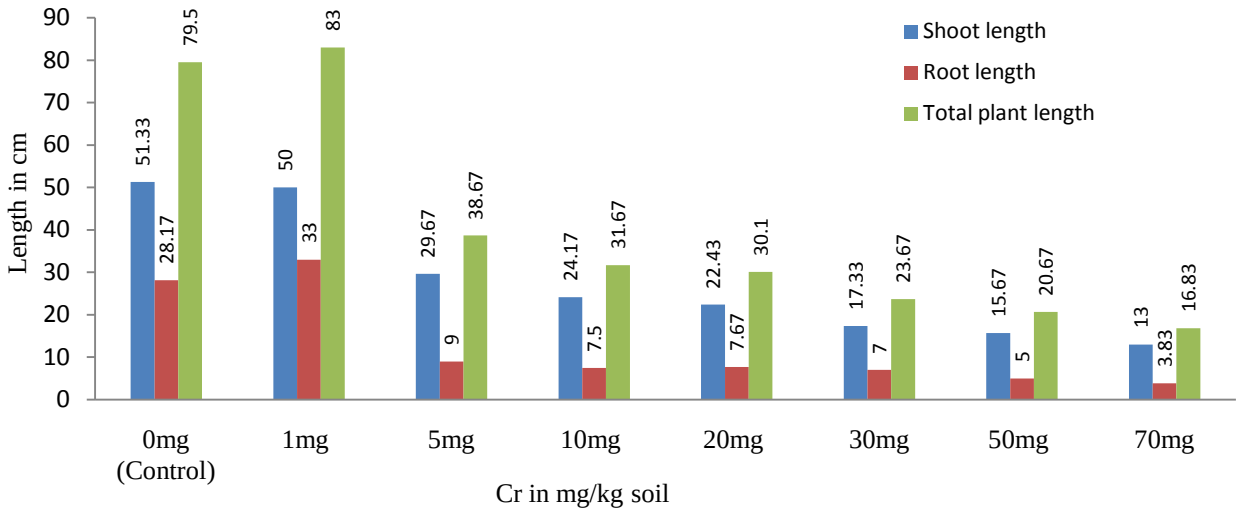

Figure 1. Effects of Cr^{VI} on *Amaranthus dubius* shoot, root and total plant length

Table 1. Duncan Analysis of Shoot Length Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05				
	1	2	3	4	5
70mg	13.00				
50mg	15.67	15.67			
30mg	17.33	17.33	17.33		
20mg		22.43	22.43	22.43	
10mg			24.17	24.17	
5mg				29.67	
1mg					50.00
0mg (C)					51.33
Sig.	.253	.081	.079	.064	.705

Means for groups in homogeneous subsets are displayed.

The difference in shoot and total plant length obtained in 1mg Cr^{VI}/kg soil treated plants over control did not differ significantly but differed significantly higher (0.05 level) over the values of rest of the treated plants. The highest reduction in shoot and total plant length obtained in 70mg Cr^{VI} differed significantly (0.05 level) over control and plants treated with 1mg, 5mg, 10mg and 20mg Cr^{VI} while the differences over 30mg and 50mg Cr^{VI} are not significant (Table 1 & Table 3). The highest root length obtained in plants treated with 1mg Cr^{VI} is significantly higher (0.05 level) over control and other treated plants while the lowest root length obtained with 70mg Cr^{VI} treatment insignificantly differed over plants treated with 50 mg and 30mg Cr^{VI} but significantly lower (0.05 level) over rest of the treated plants (Table 2). Cr ^{VI} stress has been reported to decrease the root growth in *Pisum sativum* [8]. The root is the part of the plant which is in direct contact with growing medium and the Cr^{VI} enters the root system along with carrier elements like suphate, iron etc and accumulates in higher concentration compared to other regions of the plant due to immobilization of Cr^{VI} in vacuoles of root cells [9]. This toxic concentration in the root cells may disturb cell division and elongation and may also cause imbalances in Ca^{2+} transport between plasma membrane and cytoplasm [10]. These factors might have contributed to reduction in root length and overall growth of root system. The reduced root growth can also be attributed to toxicity induced extension of cell cycle in the roots [11]. The chromium toxicity induced inhibition of shoot length as well as total plant length is observed and reported previously in *Oryza sativa* L. [12]. The present study inferences are also in similar lines with these reports. The reduced uptake of water and minerals and their poor mobilization to shoot system imposed by poor root system development, root injury and suberization of root under chromium stress could be the result of reduced shoot growth over control plants [13,14,15].

Table 2. Duncan Analysis of Root Length Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05				
	1	2	3	4	5
70mg	3.83				
50mg	5.00	5.00			
30mg	7.00	7.00	7.00		
10mg		7.5.	7.5		
20mg			7.67		
5mg			9.00		
0mg (C)				28.17	
1mg					33.00
Sig.	.060	.119	.235	1.00	1.00

Means for groups in homogeneous subsets are displayed.

Table 3. Duncan Analysis of Total Plant Length Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05			
	1	2	3	4
70mg	16.83			
50mg	20.67			
30mg	23.67	23.67		
20mg		30.10	30.10	
10mg		31.67	31.67	
5mg			38.67	
0mg (C)				79.50
1mg				83.00
Sig.	.117	.070	.054	.385

Means for groups in homogeneous subsets are displayed.

3.2. Total Leaf Number and Leaf Area/Per Plant

The analysis of the data shown in Figure 2 and Figure 3 revealed a progressive reduction in total leaf number and leaf area/plant in *A. dubius* along with the increase in concentration of Cr^{VI} treatments, with the highest values being recorded in 1mg Cr^{VI} treatment while the lowest values in the highest concentration treatment of 70mg Cr^{VI}. The differences in leaf number caused due to 1mg and 5mg Cr^{VI} treatment did not differ significantly over the control while the differences obtained in all other concentration treatments are significantly lower. Similarly, the average leaf area/plant of 345.74 cm^2 obtained in 1mg Cr^{VI} treatment insignificantly differed over control value of 362.12 cm^2 while values of others are significantly lower (Table 4 & Table 5).

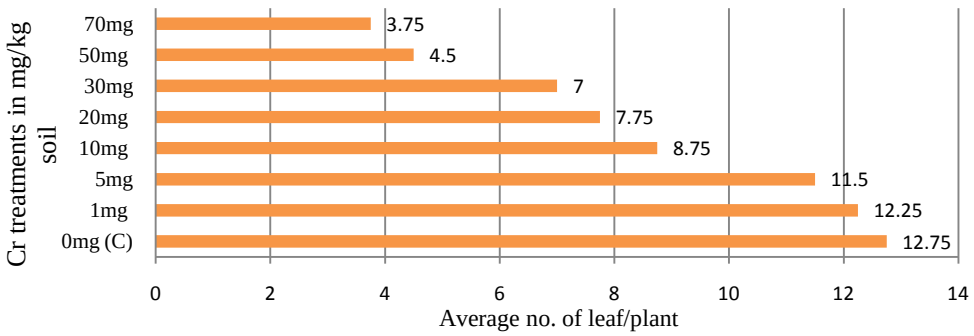

Figure 2. Effects of Cr^{VI} treatments on total number of leaf/plant in *Amaranthus dubius*

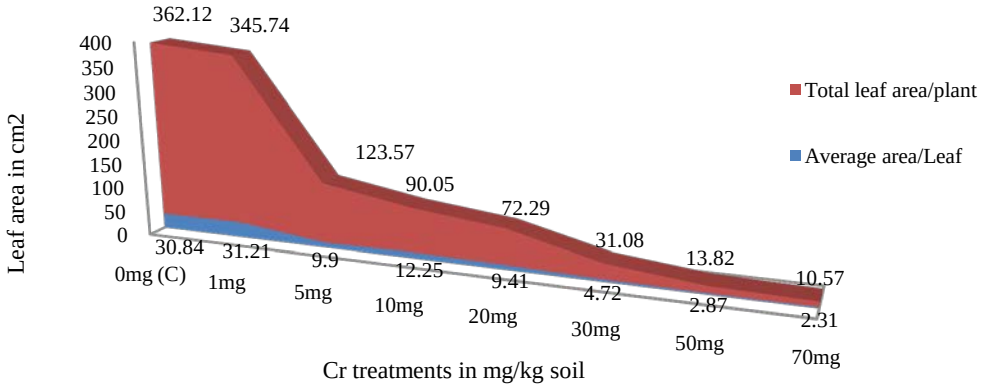

Figure 3. Effects of different concentrations of Cr^{VI} on leaf area/plant in *Amaranthus dubius*

Table 4. Duncan Analysis on the Total Number of Leaf/Plant in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05			
	1	2	3	4
70mg	3.75			
50mg	4.50			
30mg		7.00		
20mg		7.75	7.75	
10mg			8.75	
5mg				11.50
1mg				12.25
0mg (C)				12.75
Sig.	.334	.334	.201	.132

Means for groups in homogeneous subsets are displayed.

Table 5. Duncan Analysis on the Total Leaf Area/Plant in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05			
	1	2	3	4
70mg	10.57			
50mg	13.82			
30mg	31.08	31.08		
20mg	72.29	72.29	72.29	
10mg		90.05	90.05	
5mg			123.57	
1mg				345.74
0mg (C)				362.12
Sig.	.066	.070	.113	.585

Means for groups in homogeneous subsets are displayed.

The leaf morphological as well as physiological characteristics of plants are generally highly sensitive to heavy metal stress and the major visible impacts include reductions in leaf parameters. It is reported that reduction in leaf number and leaf area in *Brassica juncea* under Cr^{VI} stress and could be used as an indicator of heavy metal toxicity [16]. Similar observation is also reported in *Citrullus vulgaris* exposed to Cr^{VI} stress [17]. The inferences of the present study are in agreement with these reports and further revealed the reductions in leaf parameters are more or less progressive and positively correlated with increase in Cr^{VI} stress. The reductions in the leaf number and leaf area of Cr^{VI} treated plants over the control could be due to the inhibitory effect of chromium toxicity on two major physiological processes, cell division and expansion, which in turn can be attributed to the adverse effects on hydraulic conductance and water potential of plant tissues and restrictions in the uptake of mineral nutrients like nitrate, potassium etc [18,19,20].

3.3. Fresh and Dry Weight of Shoot, Root and Total Plant

The Cr^{VI} treatments in *A. dubius* plants caused differences in the fresh weight of shoot and root components and in the total plant over control (Figure 4). The fresh weight decreased progressively over the control as the concentration of treatment increased. The highest decrease of 97.08% for shoot component, 97.66% for root component and 97.16% for total plant over control is obtained with 70mg $Cr^{(VI)}$ treatment while the lowest decrease of 10.53%, 14.06% and 10.99% is obtained respectively for the shoot, root and total plant in 1mg Cr^{VI} treatment**.** A similar trend is also obtained with dry weight measurements where the highest reduction of 98.19%, 97.96% and 98.16% recorded respectively for shoot, root and total plant and the lowest reduction of 9.99%, 14.74% and 10.52% recorded respectively for shoot, root and total plant (Figure 5). The differences in the fresh biomass accumulation of shoot component as well as the total plant obtained in 1mg Cr^{VI} treatment over the control plants are not significant but significantly higher over the values of rest of the treated plants (Table 6 & Table 8). The decreases in the root fresh biomass in all Cr^{VI} treated plants are significantly lower over control (Table 7). The lowest shoot fresh biomass recorded by 70mg Cr^{VI} treatment is significantly lower over 1mg, 5mg and control treatments while that of root component as well as the total plant differed significantly lower only over control and 1mg Cr^{VI} treatments.

Table 6. Duncan Analysis of Shoot Fresh Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05		
	1	2	3
70mg	.99		
50mg	2.15	2.15	
30mg	3.43	3.43	
20mg	4.45	4.45	
10mg	4.53	4.53	
5mg		6.37	
1mg			30.33
0mg (C)			33.90
Sig.	.162	.099	.122

Means for groups in homogeneous subsets are displayed.

Table 7. Duncan Analysis of Root Fresh Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05		
	1	2	3
70mg	.12		
50mg	.18		
30mg	.25		
20mg	.31		
10mg	.35		
5mg	.52		
1mg		4.40	
0mg (C)			5.12
Sig.	.282	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 8. Duncan Analysis of Total Plant Fresh Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05	
	1	2
70mg	1.11	
50mg	2.35	
30mg	3.68	
20mg	4.76	
10mg	4.88	
5mg	6.89	
1mg		34.73
0mg (C)		39.02
Sig.	.053	.103

Means for groups in homogeneous subsets are displayed.

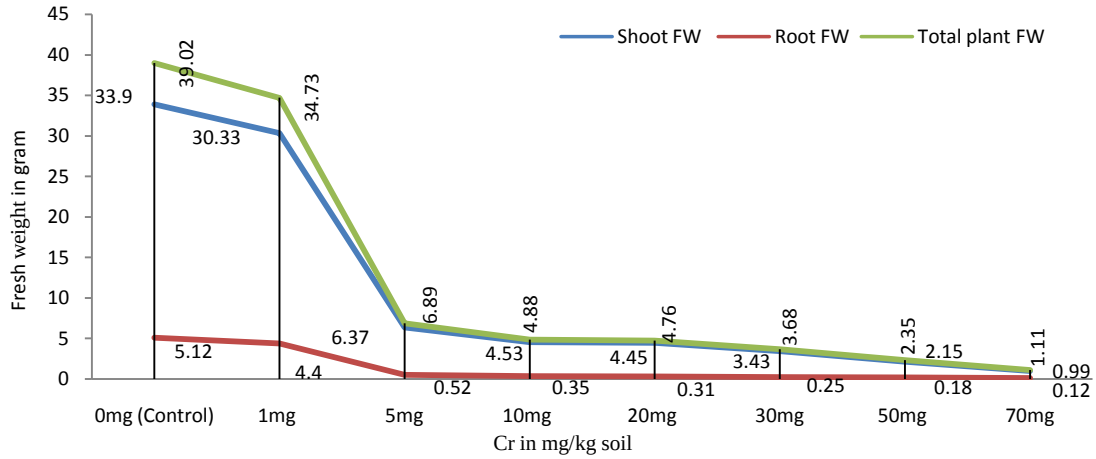

Figure 4. Effects of Cr^{VI} on the fresh weight of *Amaranthus dubius* shoot, root and total plant

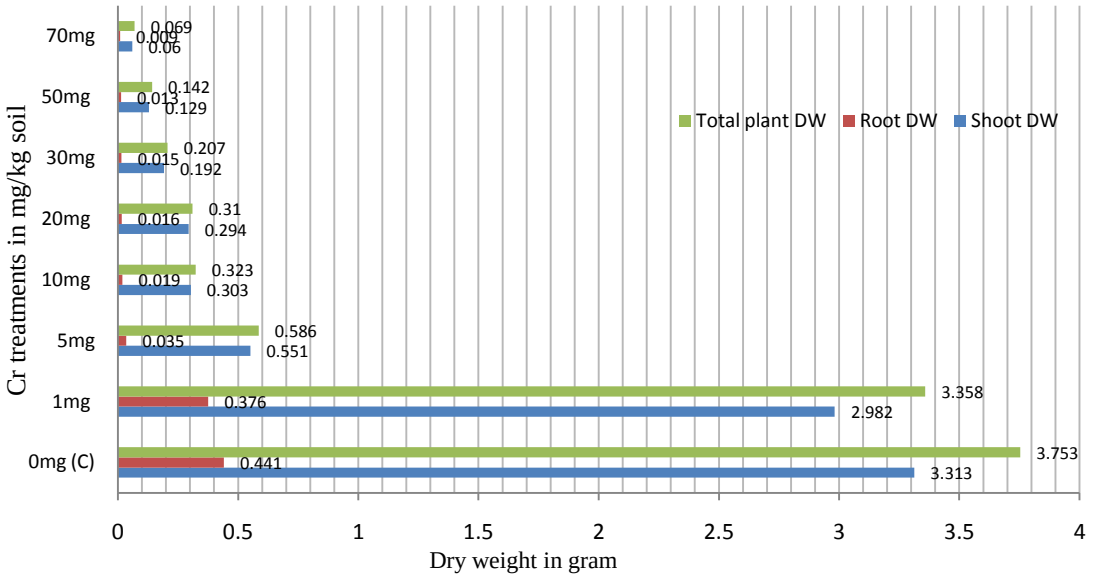

Figure 5. Effects of Cr^{VI} on the dry weight of *Amaranthus dubius* shoot, root and total plant

The reduction in dry biomass of shoot component and total plant in 1mg Cr^{VI} treated plants showed no significant differences over control while the reductions in other treated plants are significant over control and 1mg Cr^{VI} treated plants. With respect to root biomass, the reductions in all the treatments are significant over control. The highest decrement recorded by 70mg Cr^{VI} treatment showed no significant differences over 50mg, 30mg, 20mg, 10mg and 5mg Cr^{VI} treatments (Table 9, Table 10 & Table 11).

The Cr toxicity has been reported to reduce biomass of leaves, shoot, root, and total plant in *Helianthus annuus* [21]. The major factors responsible for the decreased fresh and dry biomass of shoot, root and total plant of *A. dubius* in the study might be due to Cr^{VI} toxicity induced water stress, restricted availability of essential nutrients like Zn, Mn, Cu, Fe, Na and Ca, reduction in photosynthetic pigments etc. which lead to reduced growth rate under disturbed cell cycle and cell division [22,23].

Table 9. Duncan Analysis of Shoot Dry Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05	
	1	2
70mg	.0597	
50mg	.1290	
30mg	.1917	
20mg	.2943	
10mg	.3033	
5mg	.5510	
1mg		2.9820
0mg (C)		3.3113
Sig.	.058	.146

Means for groups in homogeneous subsets are displayed.

Table 10. Duncan Analysis of Root Dry Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05		
	1	2	3
70mg	.009		
50mg	.013		
30mg	.015		
20mg	.016		
10mg	.019		
5mg	.035		
1mg		.376	
0mg (C)			.441
Sig.	.418	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 11. Duncan Analysis of Total Plant Dry Weight Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05	
	1	2
70mg	.069	
50mg	.142	
30mg	.207	
20mg	.310	
10mg	.323	
5mg	.586	
1mg		3.358
0mg (C)		3.753
Sig.	.073	.122

Means for groups in homogeneous subsets are displayed.

3.4. Root/Shoot Weight Ratio (R/S Weight Ratio)

The Cr^{VI} treatments have caused a reduction in R/S ratio in terms of both fresh weight (FW) and dry weight (DW) over control, with the exception of R/S dry weight ratio in 70mg Cr^{VI} which recorded an insignificant increase of 11.36% over control (Figure 6). The results generally indicate that the reductions are progressive up to 20mg Cr^{VI} treatment and then progressive enhancement up to 70mg Cr^{VI}. The highest reduction of 55.61% of fresh weight ratio and 60.29% of dry weight ratio are recorded in 20mg Cr^{VI} treatment over control while the lowest reduction of 2.99% for fresh weight and 4.14% for dry weight are recorded in 1mg Cr^{VI} treatment. With the exception in 1mg Cr^{VI} treated plants with respect to R/S fresh weight ratio and 1mg and 70mg Cr^{VI} treated plants with respect to R/S dry weight ratio, the decrease in R/S weight ratio of all the treated plants differ significantly lower over control (Table 12 & Table 13). Generally, each plant type has an inherent normal R/S ratio under healthy environmental conditions and it clearly indicates normal balanced growth of the plant, but any decrease in the R/S ratio from the normal level indicates that plant has encountered some kind of stresses. The root system is the part which is directly in contact with the toxic soil environment and as the Cr^{VI} metal is toxic and non-essential for the plant, it is getting sequestered in vacuoles of root cells which in turn may cause disturbed cell division and elongation of root cells followed by the decline in the root growth and impairment of the root penetration leading to reduction in R/S ratio [24]. However after certain level of Cr^{VI} concentration, the further enhancement caused a gradual increase from the lowered R/S ratio due to initial level concentrations. This might be due to stunned shoot growth which is as a result of severe Cr^{VI} toxicity induced root injury. The root injury may cause considerable reduction in absorption as well as translocation of root-zone resources such as water and nutrients into the shoot system for their utilization into above ground biomass yield by making use of CO_2 and sunlight.

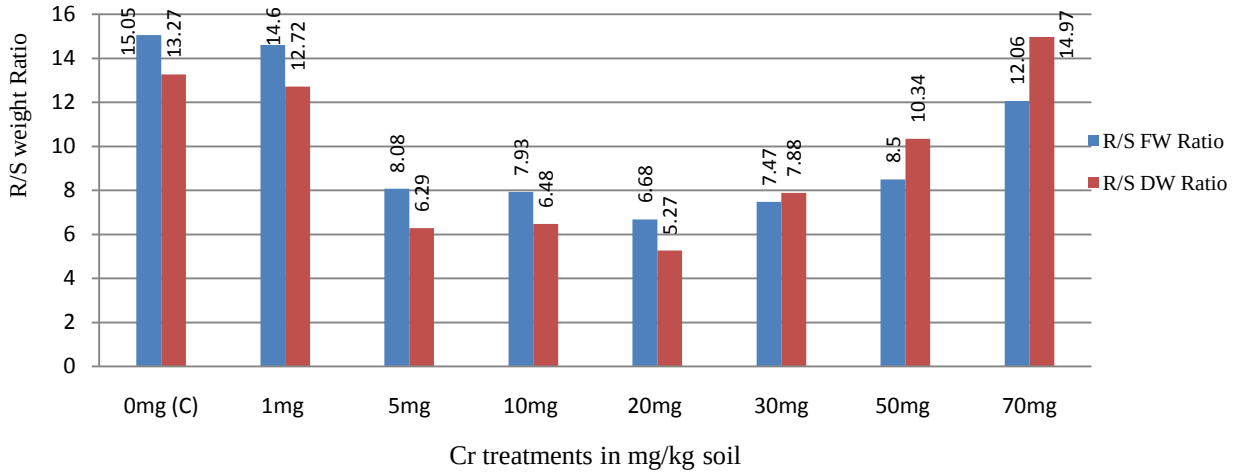

Figure 6. Effects of different concentrations of Cr^{VI} on Root/Shoot fresh and dry weight ratio in *Amaranthus dubius*

Table 12. Duncan Analysis of R/S Fresh Weight Ratio Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05		
	1	2	3
20mg	6.68		
30mg	7.47		
10mg	7.93		
5mg	8.08		
50mg	8.50		
70mg		12.06	
1mg			14.60
0mg (C)			15.05
Sig.	.098	1.000	.638

Means for groups in homogeneous subsets are displayed.

Table 13. Duncan Analysis of R/S Dry Weight Ratio Variation in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05			
	1	2	3	4
20mg	5.27			
5mg	6.29	6.29		
10mg	6.48	6.48		
30mg		7.88		
50mg			10.34	
1mg				12.72
0mg (C)				13.27
70mg				14.97
Sig.	.295	.173	1.000	.060

Means for groups in homogeneous subsets are displayed.

Table 14. Tolerance Index (TI %) and Inhibition of Plant Growth (IPG %) in *Amaranthus dubius* Treated with Cr^{VI}

Treatment Cr^{VI} / kg soil	Tolerance index (TI %)				Inhibition of plant growth (IPG %)			
	Fresh biomass	SD±	Dry biomass	SD±	Fresh biomass	SD±	Dry biomass	SD±
0mg (C)	100.00	0.0	100.00	0.0	0.00	0.0	0.00	0.0
1mg	88.92	2.7	89.39	2.4	11.11	3.3	10.61	2.9
5mg	17.64	0.28	15.65	0.5	82.37	0.4	84.36	0.6
10mg	12.18	3.5	8.45	2.3	87.80	4.2	91.61	2.8
20mg	12.07	1.6	8.17	1.1	87.97	1.9	91.86	1.4
30mg	9.39	0.8	5.50	0.6	90.63	1.0	94.50	0.7
50mg	6.07	0.5	3.82	0.3	93.93	0.7	96.94	1.6
70mg	2.89	0.5	1.86	0.4	97.07	0.6	98.13	0.5

3.5. Tolerance Index (TI %) and Inhibition of Plant Growth (IPG in %)

The details of tolerance index (TI %) and inhibition of plant growth (IPG %) in terms of fresh and dry biomass accumulation in *A. dubius* under Cr^{VI} stress is depicted in the Table 14. The highest TI of 88.92% and 89.39% and the lowest IPG of 11.11% and 10.61% respectively in terms of plant fresh biomass and dry biomass in the study is obtained in 1mg Cr^{VI} treatment. The data revealed that the further increase in Cr^{VI} stress is causing a progressive decline in the TI % and a corresponding progressive enhancement in IPG % and finally the lowest TI of 2.89% and 1.86% and likewise the maximum IPG of 97.07% and 98.13% respectively obtained in the highest concentration of 70mg Cr^{VI}. The decrease in the tolerance index and the increase in the inhibition of plant growth recorded in all the Cr^{VI} treated plants differed significantly over control, both in terms of fresh and dry biomass accumulation (Table 15, Table 16, Table 17 & Table 18). The highest TI as well as the lowest IPG recorded by plants treated with 1mg Cr^{VI} significantly differed over other treated plants. Similarly, the lowest TI recorded by 70mg Cr^{VI} treated plants are significantly lower compared to others while the highest IPG value obtained by these plants is insignificant over 50mg Cr^{VI} treated plants but significant over rest of the treated plants.

The inferences obtained from the analysis of different morphological growth parameters in the study clearly indicated reduction in tolerance index and inhibition of growth and this could be due to Cr^{VI} toxicity induced damage and poor development of root system, which causes restricted absorption of water and essential elements and their poor mobilization into different regions of plants to carryout various physiological growth processes to yield biomass. The study revealed the existence of a positive correlation between the inhibition of growth and the intensity of Cr^{VI} stress. This can be attributed to the differences in Cr^{VI} toxicity and that induced extension of cell cycle and related inhibition in cell division and enlargement which in turn may be due to the differences in alteration or imbalance in physiological processes. The Cr^{VI} toxicity can disturb biosynthesis of photosynthetic pigments, reduction in stomatal conductance and gas exchange activities, disruption in electron transport system etc which are capable of undesirably affecting the rate of photosynthesis, ATP production and energy level of cells etc.

Table 15. Duncan Analysis of Tolerance Index Based on the Fresh Biomass in *Amaranthus dubius* Plants Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
70mg	2.89						
50mg		6.07					
30mg			9.39				
20mg				12.07			
10mg				12.18			
5mg					17.64		
1mg						88.92	
0mg (C)							100.00
Sig.	1.000	1.000	1.000	.930	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 16. Duncan Analysis of Tolerance Index Based on the Dry Biomass in *Amaranthus dubius* Plants Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05					
	1	2	3	4	5	6
70mg	1.86					
50mg		3.82				
30mg		5.50				
20mg			8.17			
10mg			8.45			
5mg				15.65		
1mg					89.39	
0mg (C)						100.00
Sig.	1.000	.076	.759	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table 17. Duncan Analysis of Inhibition of Plant Growth (%) in Terms of Fresh Biomass in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05					
	1	2	3	4	5	6
0mg (C)	0.00					
1mg		11.11				
5mg			82.37			
10mg				87.80		
20mg				87.97		
30mg				90.63	90.63	
50mg					93.93	93.93
70mg						97.07
Sig.	1.000	1.000	1.000	.129	.068	.081

Means for groups in homogeneous subsets are displayed.

Table 18. Duncan Analysis of Inhibition of Plant Growth (%) in Terms of Dry Biomass in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05					
	1	2	3	4	5	6
0mg (C)	0.00					
1mg		10.61				
5mg			84.36			
10mg				91.61		
20mg				91.86		
30mg				94.50	94.50	
50mg					96.94	96.94
70mg						98.13
Sig.	1.000	1.000	1.000	.059	.092	.393

Means for groups in homogeneous subsets are displayed.

3.6. Net Photosynthetic Rate (P_N)

The highest average net photosynthetic rate of 10.08µmol CO_2m^2/sec obtained in *A. dubius* plants treated with lowest concentration of 1mg Cr^{VI}. This is about 8.83% insignificant increases over control while the increase over rest of the Cr^{VI} treated plants is significant. However, further increase in Cr^{VI} stress caused progressive reduction in the P_N value and the lowest P_N value of 2.66µmol CO_2m^2/sec is obtained in the highest concentration of 70mg Cr^{VI}, which is about 71.06% decrease over control (Figure 7). This lowest P_N value has no significant differences over the P_N value obtained in 50mg and 30mg Cr^{VI} stress while differed significantly lower over control and rest of the Cr^{VI} stress (Table 19).

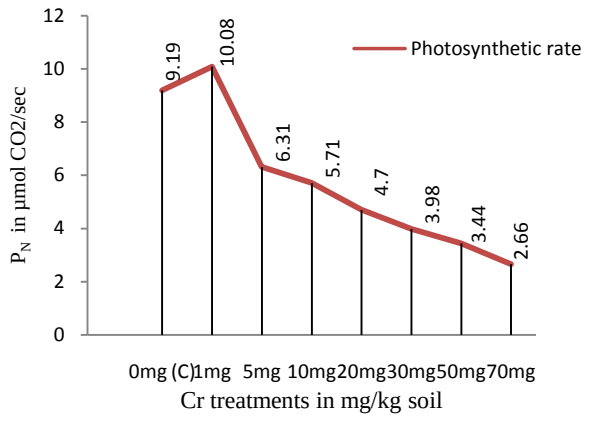

Figure 7. Rate of photosynthesis in *Amaranthus dubius* against different concentrations of Cr^{VI} stress

Table 19. Duncan Analysis of Net Photosynthetic Rate in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05				
	1	2	3	4	5
70mg	2.66				
50mg	3.44	3.44			
30mg	3.98	3.98			
20mg		4.70	4.70		
10mg			5.71	5.71	
5mg				6.31	
0mg (C)					9.197
1mg					10.08
Sig.	.064	.077	.134	.365	.186

Means for groups in homogeneous subsets are displayed.

There are reports on the inhibition of growth and productivity in plants due to heavy metal stress induced disturbances in electron transport system and reduction in net photosynthetic rate [25,26]. Previous studies have recorded $Cr^{(VI)}$ toxicity induced depletion of net photosynthetic rate in *Lolium perenne* [27] and in *Oryza sativa* [28]. The observations of the present study are in agreement with these reports. The reduction in net photosynthetic rate and growth recorded in *A. dubius* plants subjected to Cr^{VI} stress can be attributed to their inhibitory action on ATP production and energy level of cells by affecting the primary photosynthetic reactions. This might be due to the disturbances on electron carriers and the flow rate of electron from water to PS-II reaction centre by Cr^{VI} toxicity.

3.7. Stomatal Conductance (g_S)

The stomatal conductance of *A. dubius* under Cr^{VI} stresses are depicted in Figure 8. The highest stomatal conductance in the study is recorded in plants treated with the lowest Cr^{VI} stress while the maximum reduction in the highest Cr^{VI} stress and the data revealed that the reductions are progressive from the highest stomatal conductance of 0.077 m mol $m^{-2}s^{-1}$ obtained in the study towards the lowest value of 0.023 m mol $m^{-2}s^{-1}$, along with the increases in Cr^{VI} stress. The increase in stomatal conductance of 2.6% recorded by plants treated with 1mg Cr^{VI} over control is insignificant while its differences over other Cr^{VI} treated plants are significantly higher. Similarly, the lowest stomatal conductance recorded by 70mg Cr^{VI} treated plants did not differ significantly over 50mg Cr^{VI} treatment whereas the differences over rest of the treated plants are significantly lower (Table 20). While evaluating the impact of Cr stress on fluorescence parameters, the decrease in stomatal conductance followed by fall in the rate of CO_2 assimilation reported earlier in plants [29] and these observations are in similar lines with present study inferences. The reduction in stomatal conductance under Cr^{VI} stress inferred in the study might be the result of toxicity induced water imbalance and poor stomatal opening caused by loss in turgor pressure inside the guard cells of stomata [30]. The production and accumulation of abscisic acid (ABA) molecules following water stress and their involvement in signal pathways to control water loss by stomatal closure might be the key factor in the decrease of stomatal conductance.

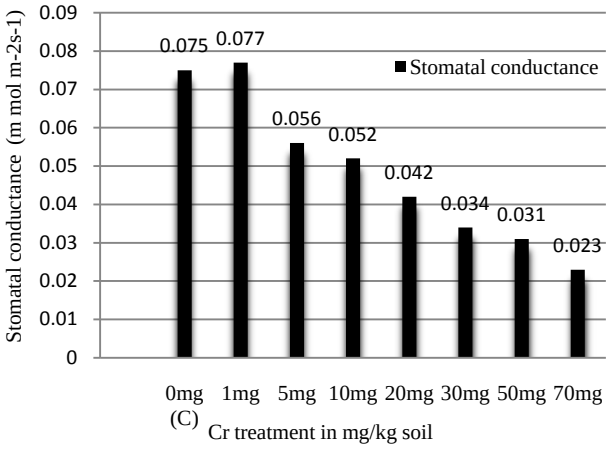

Figure 8. Stomatal conductance in *Amaranthus dubius* against different concentrations of Cr^{VI} stress

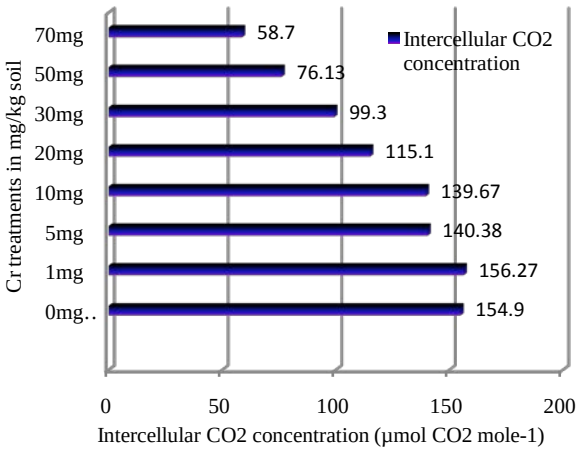

Figure 9. Intercellular CO_2 concentration in *Amaranthus dubius* against different concentrations of Cr^{VI} stress

Table 20. Duncan Analysis of Stomatal Conductance in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05				
	1	2	3	4	5
70mg	.023				
50mg	.031	.031			
30mg		.034			
20mg			.042		
10mg				.052	
5mg				.056	
0mg (C)					.075
1mg					.077
Sig.	.056	.536	1.000	.251	.566

Means for groups in homogeneous subsets are displayed.

Table 21. Duncan Analysis of Intercellular CO_2 Concentration in *Amaranthus dubius* Treated with Cr^{VI}

Cr^{VI} Treatment	Subset for alpha = 0.05			
	1	2	3	4
70mg	58.70			
50mg	76.13	76.13		
30mg		99.30	99.30	
20mg			115.10	
10mg				139.67
5mg				140.38
0mg (C)				154.90
1mg				156.27
Sig.	.145	.055	.186	.205

Means for groups in homogeneous subsets are displayed.

3.8. Intercellular CO_2 Concentration (C_i)

The intercellular CO_2 concentration (C_i) levels in *A. dubius* plants under Cr^{VI} stresses are found to respond in similar lines with stomatal conductance and net photosynthetic rate (Figure 9). The C_i value of 156.27 µmol CO_2 $mole^{-1}$ recorded in 1mg Cr^{VI} treated plants is about 0.88% increase over control. Further enhancement in Cr^{VI} stress caused a progressive reduction and the lowest C_i value of 58.7µmol CO_2 $mole^{-1}$ recorded in 70mg Cr^{VI} treated plants is about 62.10% decrease over control. The C_i values recorded by plants treated with 1mg, 5mg and 10mg Cr^{VI} insignificantly differed among themselves and over control plants while they differed significantly higher over the values of 20mg, 30mg, 50mg and 70mg Cr^{VI} treated plants. The lowest C_i value recorded by 70mg Cr^{VI} treated plants did not significantly differ over 50mg Cr^{VI} treated plants while the differences over rest of the treated plants are significant (Table 21). Cr^{VI} stress has been reported to negatively interfere with assimilation of CO_2 and which affects intercellular CO_2 concentration and net photosynthetic rate in *Triticum aestivum* L. [31]. The present study indicates the existence of a negative correlation between the degree of Cr^{VI} stress and C_i value, which is evident from the progressive reduction in CO_2 concentrations along with the increasing Cr^{VI} stress. The decrease of C_i value clearly revealed the toxic and inhibitory effect of Cr^{VI} stress on stomatal conductance, which affects the essential gas exchange activities [32].

4. Conclusion

The present investigation clearly revealed the hexavalent chromium (Cr^{VI}) stress has significantly deleterious effects on the morphological growth parameters and photosynthesis related characteristics like net photosynthetic rate, stomatal conductance and intercellular CO2 concentration in *Amaranthus dubius* plants. The reduced water potential and enhanced diffusive resistance due to root injury and poor root system development induced by Cr^{VI} toxicity might have led to the restrictions in the availability of water and essential elements necessary for relevant physiological growth processes. This could have contributed to the disruption in mitochondrial electron transport, oxidative stress, chloroplast structural alteration, inhibition of chlorophyll biosynthesis, reduction in stomatal conductance and intercellular CO_2 concentration which ultimately lead to decreased net photosynthetic rate and reduced biomass yield. Although several literatures are available to understand the interaction of toxic Cr^{VI} with essential metal ions which are relevant in biosynthetic and physiological pathways in plants, their involvement and mechanism of causing toxicity at bio-molecular level is still poorly understood.

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OP-124

Genotoxic and cytotoxic effects of hexavalent chromium (Cr^{VI}) stress in *Amaranthus dubius* Mart. ex Thell.

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Industrialization, urbanization and unscientific disposal of waste is a major problem that increases the heavy metal contamination in the soil and water resources. Chromium^{VI} ranks high as an industrial pollutant and toxic due to its non-biodegradable property. *Amaranthus dubius* is known as poor man's vegetable is a bioaccumulator of chromium. Chromium is a heavy metal can influence the genetical and cytological characteristics and hence its effects on *A. dubius* are a study that requires investigation. The study aimed at the evaluation of genotoxic and cytotoxic effect of hexavalent chromium on the root tip cells of *Amaranthus dubius* Mart. ex Thell. Healthy seeds of *Amaranthus dubius* were treated with different concentrations of hexavalent chromium (Cr^{VI}) 1mg, 10mg, 30mg, 50mg, 70mg and 0.0mg Cr^{VI}/litre, prior to germination and genotoxic and cytotoxic studies are carried out in the root tip cells of germinated seeds. The percentage of dividing cells and mitotic index in the root tip cells of *Amaranthus dubiustreated* with various concentrations of Chromium^{VI} tend to reduce with the exception of 1mg Cr^{VI}/ litre. The percentage of chromosomal aberration increases up to 20mg Cr^{VI}/litre and then tend to decrease with higher concentrations while overall percentage of abnormality tend to increase with increasing in concentration. Various chromosome abnormalities observed in the study are misorientaton, clumping, stickiness, fragmentation, pulverization and prophase disturbances while non-chromosomal abnormalities include nuclear lesions, binucleate cells, nuclear bulging, micronuclei, ghost cells and misplaced nucleus. The genotoxic and cytotoxic studies on *Amaranthus dubius* provided a warning about the toxicity of Cr^{VI} and make us aware on the need to be inquisitive on the source of vegetables being used for human consumption

OP-125

The Impact of Chromium (Cr) Stress on the Structural Integrity, Antioxidant Enzyme Activity and Growth in *Amaranthus dubius* Mart.ex Thell.

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Introduction

Environmental pollution due to heavy metals is a common problem in the field of agricultural production in many countries. Chromium is used on a large scale in many industries including metallurgy, electroplating, production of paints and pigments, tanning, wood preservation, chemical production, and pulp and paper production[1] and this industrial activity have led to the widespread contamination of metal chromium and have increased its bioavailability and biomobility. In nature, Cr exists in two different stable oxidation states; trivalent (Cr^{III}) and hexavalent (Cr^{VI}) chromium. Both Cr^{III} and Cr^{VI} differ in terms of mobility, bioavailability and toxicity. Hexavalent chromium (Cr^{VI}) is considered the most toxic form of Cr as it is a strong oxidant with a high redox potential in the range of 1.33-1.38 eV accounting for a rapid and high generation of ROS and its resultant toxicity[2]. The aim of the present study was to investigate the effect of different concentrations of hexavalent chromium on various growth parameters, structural integrity and antioxidant enzymes of *Amaranthus dubius* Mart ex. Thell.

Materials and Methods

Healthy seeds of *Amaranthus dubius* collected from Kerala Agricultural University, Thrissur is subjected to germination. Healthy germinated seeds which emerge out of the growing media are transferred carefully into pots containing standard potting mixture prepared by using farmyard manure, red soil and sand in the ratio 1:1:1. One week after transplantation, healthy uniform sized and aged seedlings are selected in triplicate blocks of 30 each for each treatment. Treatments include eight different specific concentration levels of heavy metal chromium which are selected based on a preliminary germination study. Different concentrations include 1mgCr/Kg potting mixture, 10mgCr/Kg potting mixture, 30mgCr/Kg potting mixture, 50mgCr/Kg potting mixture,

70mgCr/Kg potting mixture and 0.00mg Cr/ Kg potting mixture (control). Different concentrations of metal chromium are prepared from potassium dichromate salt (K2Cr2O7) of analytical reagent grade. The required concentrations of chromium are applied to the potting mixture as aqueous solution in such a way that every 200ml contains required amount of chromium. 200ml of distilled water alone is used as control (0.0mgCr/200ml). Anatomical, physiological and morphological parameters were analysed for all the Cr treated as well as the control plants of *Amaranthus dubius,* to determine the impact of different level of Cr stress.

Analysis of morphological growth parameters

Morphological parameters like shoot length, root length, plant height, fresh and dry weight of the plant of hexavalent chromium treated and control plants of *A.dubius* were measured and compared. Root length, shoot length and seedling size of the plant were measured using a marked one meter scale. Fresh and dry weight biomass was measured using Sartorious electronic balance (MCI Analytic AC 210 P. Germany). Dry biomass was determined by placing the plant material in an oven at 80°C for 24 hours.

Analysis of anatomical parameters Transverse section of Stem and Root

Free hand thin transverse sectioning of the stem and root were made using sharp razor blade. Plant samples for various anatomical studies were collected from uniform regions of plants from all treatments at uniform growth rate. Stem sections were made from region of 4-6 cm above the collar region while root sections were made from 2-4 cm below the collar region. Sections were stained with safranin and mounted on glycerin for microscopic observations. Shape and arrangement of cells, nature of cell wall margin, structural integrity and cell damages were observed.

Photomicrography

Observations and photomicrographs of the slides of the anatomical sections were taken using Magnus MLX Series Trinocular Microscope fitted with Magnus MIPS - USB Camera (Olympus India).

Estimation of antioxidant enzymes

A) Assay of Superoxide dismutase (SOD)[3]

The sample (0.5gm), were ground with 3.0ml of potassium phosphate buffer and centrifuged at 2000g for 10 minutes and the supernatants were used for the assay. Assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of Nitroblue tetrazolium (NBT), 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

B) Assay of Catalase (CAT)[4]

20% homogenate was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay. Phosphate buffer (3.0 ml) was taken in an experimental cuvette, followed by the rapid addition of 40ul of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. The enzyme solution containing H2O2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

Result and discussion

Effect of different concentrations of hexavalent chromium (Cr^{VI}) on various growth parameters of Amaranthus dubius is depicted in Table 1. During the initial level concentration of 1mg/ Kg soil, an increase was recorded in the root length, shoot length and total plant length. However in concentrations of chromium above 1mg/Kg soil, the trend was reversed and caused reduction in growth parameters. The reduction was predominant at higher level concentrations and a maximum reduction was recorded in the highest concentrations of 70mg/Kg soil and it was a reduction of 83.44% for root length, 75.32% for shoot length and 78.13% for total plant length.

Table 1: Effects of different concentrations of hexavalent chromium (Cr^{VI}) on root length, shoot length and total plant length of *Amaranthus dubius* Mart.Ex Thell

Treatment chromium (mg/kg soil)	Average Root length (cm)	Average Shoot length (cm)	Average plant length (cm)
1mg	29.0±3.24	29.0±3.24	82.0±7.36
10mg	7.67±1.03	7.67±1.03	32.67±3.20
30mg	6.33±1.65	6.33±1.65	24.5±2.48
50gm	5.0 ±0.41	5.0 ±0.41	19.67±2.25
70mg	4.5±0.41	4.5±0.41	17.17±1.43
Control	27.17 ±3.79	27.17 ±3.79	78.5±6.96

Each mean data with SD presented in the table is an average of 4 replications

Effect of different concentrations of hexavalent chromium (Cr^{VI}) on total fresh weight and dry weight biomass in *Amaranthus dubius* is shown in Table- 2. All the treated plants exhibited a reduction in fresh weight and dry weight compared to control plants. The reduction was concentration dependant as fresh weight and dry weight biomass of *A. dubius* showed a decreasing trend with increasing concentration of Cr^{VI} in treatments. The reduction in biomass was drastic from 10mg/kg soil treatment onwards and was more prominent in 70mg/kg soil treatment with an average of 99.05% and 99.53% reduction respectively for fresh weight and dry weight over control. The present study results reveal that a concentration of hexavalent chromium above 1mg/kg soil could induce visual inhibitory effect on biomass of treated *A. dubius* plants over control.

Table 2: Effects of different concentrations of hexavalent chromium (Cr^{VI}) on total fresh weight and total dry weight of *Amaranthus dubius* Mart. Ex Thell

Treatment chromium (mg/kg soil)	Average total fresh weight (mg)	Average total dry weight (mg)
1mg	35.253±7.428	3.757±0.549
10mg	6.499±1.107	0.466±0.081
30mg	1.929±0.183	0.128±0.011
50gm	1.120±0.174	0.073±0.013
70mg	0.343±0.073	0.018±0.005
Control	36.209±5.448	3.800±0.911

Each mean data with SD presented in the table is an average of 4 replications.

The inhibition of root and shoot elongation at higher concentration of Cr^{VI} (table) can be attributed to the inhibition of mitosis, the reduced synthesis of cell-wall components, damage to the Golgi apparatus and changes in the polysaccharide metabolism due to the Cr^{VI} toxicity[5].

The reduction in growth parameters in the present study may also be due to metal toxicity induced reduction in the uptake of O2 which affect the normal physiological process favoring growth[6].

Anatomical Growth Parameters

The effect of various concentrations of hexavalent chromium (Cr^{VI}) on anatomical structures of the stem and root of *A .dubius* were analyzed. Transverse sections of stem shows that (Fig. 1), 1mg concentration of Cr^{VI} had not caused any changes in the epidermis of *A .dubius* compared to control plants where the epidermal cells were large sized, normal shaped with intact arrangement. However, from 10mg concentration treatment onwards it is observed that the epidermal cells started flattening and elongating followed by exhibiting irregularity in the cell shape and arrangement. The cells in the hypodermal layer and cortical region seems to be highly disturbed from 30mg concentration treatment onwards and the cells started exhibiting considerable variation in the size, shape and arrangement together with sinuosity in the cell wall margin. The analysis of pith cells in the transverse

sections of stem in control and 1mg hexavalent chromium treated plants showed large polygonal shaped parenchyma cells having large intercellular spaces with intact arrangement while plants grown in soil treated 'with 10mg Cr^{VI} onwards start exhibiting irregularity in the cell shape, reduction in cell size and distorted arrangement of cells. The toxic effects were more predominant at 50mg and 70mg concentration treatments. The breakdown of parenchyma tissues was also observed in 70mg Cr^{VI} treatment.

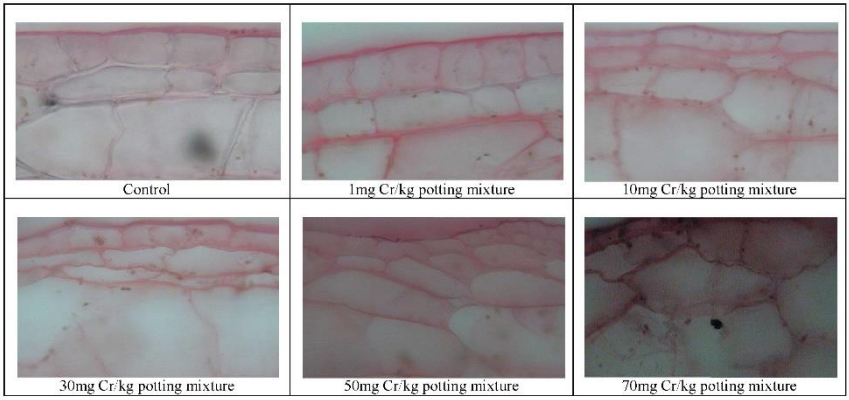

Figure 1: An enlarged portion in the stem T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing the impact of Cr^{VI} toxicity induced changes in the epidermis, hypodermis and cortex

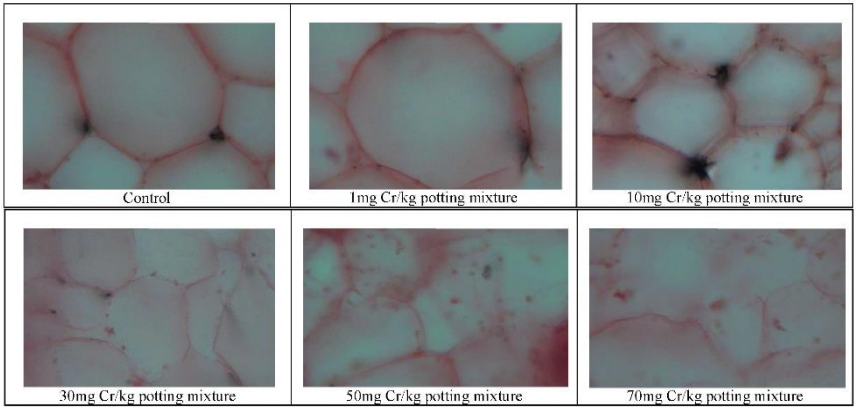

Figure 2: An enlarged portion in the stem T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing the impact of Cr^{VI} toxicity induced changes in the pith cells

Stem anatomy also reveals considerable decreasing trend in the number of vascular bundles from 10mg Cr^{VI} concentration treatment onwards and the maximum decrease was observed in the 70mg Cr^{VI} concentration treatment (Fig. 3). The secondary vascular elements form a continuous layer below the pericycle throughout in the stem of control and 1mg Cr^{VI} treated plants and then 10mg onwards the secondary vasculature gradually reduced to patches and finally the maximum reduction was observed in the stem of 70mg Cr^{VI} treated *A. dubius* plants (Fig. 3). Vessels elements of vascular bundles in the stems of 1mg Cr^{VI} treated *A. dubius* plants were observed to show structural integrity with no observable Cr^{VI} toxicity symptoms when compared to control plants. Similarly the phloem elements were also found intact with numerous cells. However from 10mg Cr^{VI} concentration treatment onwards a gradual reduction in the size and number of vessel members and phloem cells were observed and resulted in the loss of structural integrity and collapse of vessel and phloem elements due to hexavalent chromium toxicity and this toxic effects were more prominent in the stem of *A. dubius* treated with 70mg Cr^{VI} (Fig. 4).

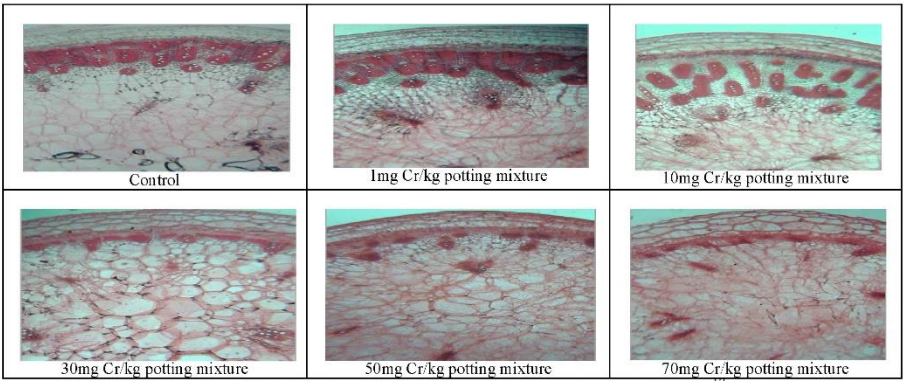

Figure 3: Stem T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing the impact of Cr^{VI} toxicity induced changes in the distribution of vascular bundles

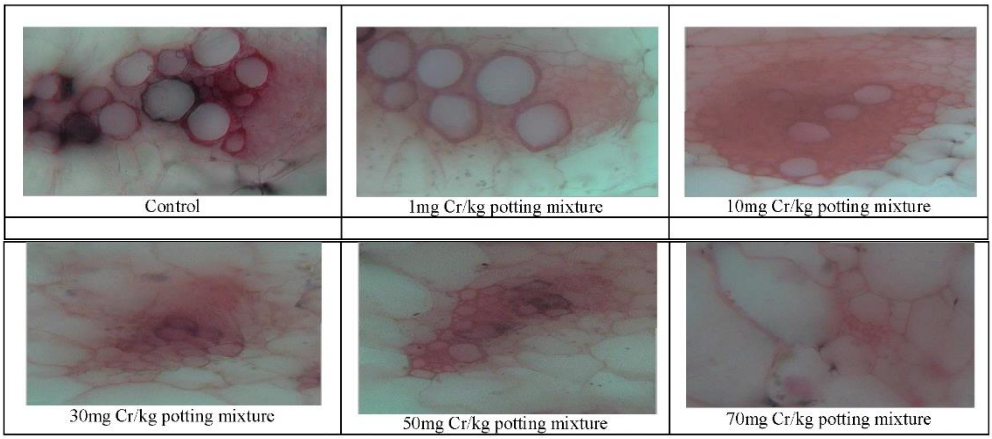

Figure 4: An enlarged portion in the stem T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing toxicity induced gradual loss of structural integrity and collapse of vessel and phloem elements.

Similar anatomical structural variations induced by hexavalent chromium toxicity in the stem of *A. dubius* were also observed in the transverse sections of root. Considerable reduction in the development of vasculature especially secondary structural elements was noticed from 10mg Cr^{VI} concentration treatment onwards (Fig. 5). In control and 1mg Cr^{VI} treated *A. dubius* plants, the cells of epidermal and cortical regions were having more or less uniform shape and size with compact arrangement and in contrast, plants treated with 10mg concentration onwards exhibited gradual disturbances and from 30mg concentration onwards cells become highly irregular in size and shape and arranged in distorted manner (Fig. 6). With respect to the cells in the pith region, as in the case of stem, in root also clear evidences for Cr^{VI} toxicity was observed as irregular shape and size of cells with wavy or curved margin (sinuosity) in the cell wall and distorted arrangement and it was more prominent in 50mg and 70mg Cr^{VI} treated *A. dubius* plants. Cell damages due to breakdown of cells in 70mg Cr^{VI} treated *A. dubius* plants were also observed (Fig. 8).

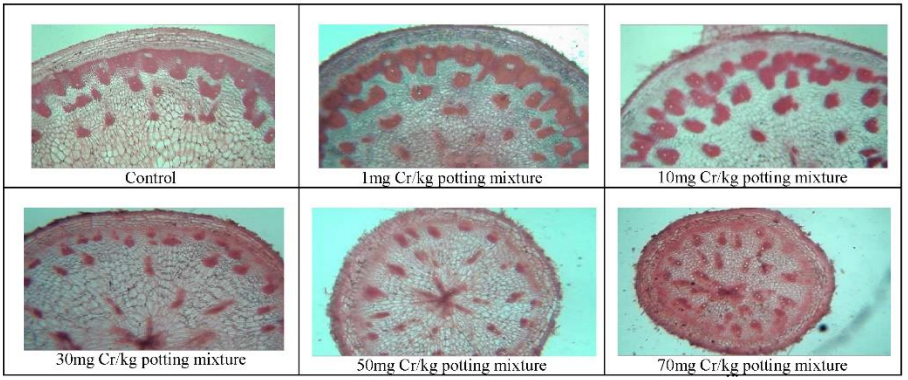

Figure 5: Root T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing the impact of CrVI toxicity on the distribution pattern of vascular bundles

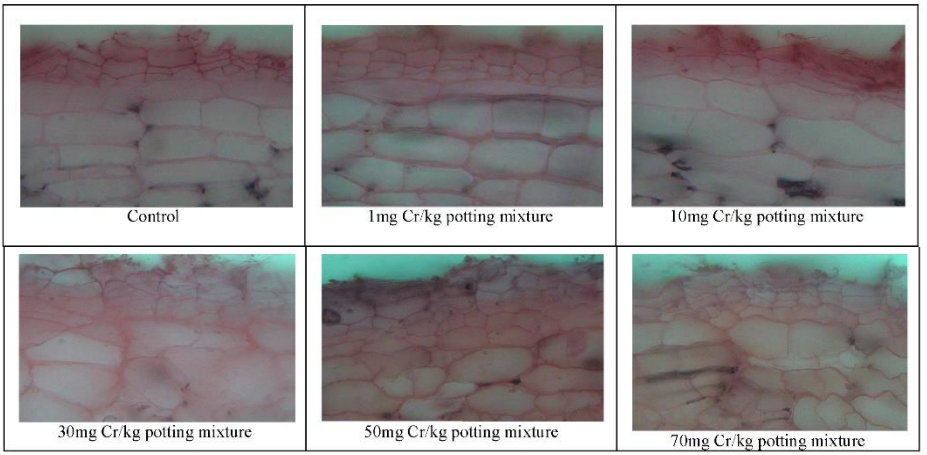

Figure 6: An enlarged portion in the root T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing Cr^{VI} toxicity induced changes in the epidermis and cortex.

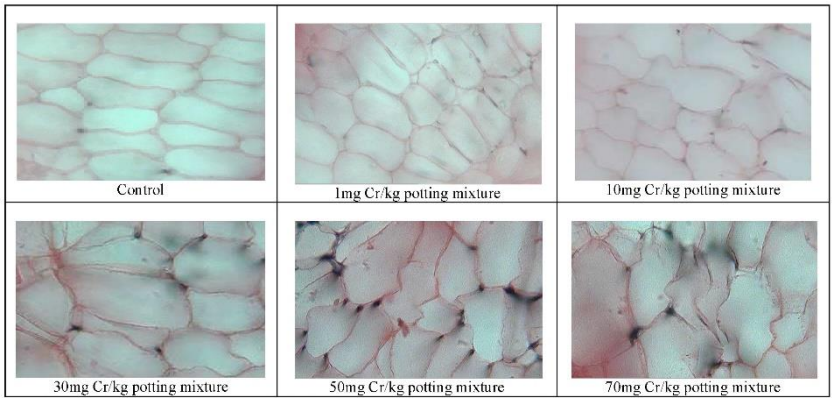

Figure 7: An enlarged portion in the root T.S of *Amaranthus dubius* treated with different concentration of hexavalent chromium (Cr^{VI}) showing the toxicity induced changes in the pith cells

The reduction in the size, irregularity in the shape and distorted arrangements of parenchymatous cells in the cortical and pith regions observed in the stem and root of *A. dubius* particularly at highest level metal toxicity are in similar lines with the observations made by Omosun et al. in *Mucuna* species grown in higher crude oil polluted soil containing heavy metals. Microscopic studies conducted in the brake fern plants by Balaji et al.[8] reveal that Cr accumulation and toxicity caused decreased intercellular spaces and break down of cells compared to the control brake fern plants and this report are in support of present observations of decreased intercellular spaces, sinuosity in the cell wall margin and cell damages observed in the parenchymatous cells in the cortical and pith region of stems and roots of *A. dubius* treated with highest concentration of 70mg Cr/ kg potting mixture. The inference obtained with respect to vasculature in response to metal toxicity in the present investigation is also in agreement with the observations and report of Clement Oluseye et al.[9] in roots and stems of *Amaranthus hybridus* where reduction in the number of vascular bundles, vessels and phloem elements at lower concentrations followed by total collapse or loss of structural integrity of the vessel and phloem elements in the extreme toxicity of the pharmaceutical effluent. The structural changes and cellular damages in the stem and root induced by hexavalent chromium (Cr^{VI}) toxicity in *A. dubius* in the present investigation can be attributed to the intense

oxidative action of Cr^{VI} [10]. The higher concentration treatments of hexavalent chromium (Cr^{VI}) may lead to an increased accumulation of Cr^{VI} in different component parts especially in the roots and may result in toxicity induced cellular plasmolysis and water stress affecting water and elemental uptake by the plant which may be the basic reason for overall decrease in growth performance of *A.dubius* as the concentration of the treatment increased.

Antioxidant enzyme activity (SOD and CAT): The activity of antioxidant enzymes catalase (CAT), and super oxide dismutase (SOD) was assessed in germinated *A. dubius* under the effect of different concentrations of Cr and the results are given in Table 3. The results of the present study showed all the tested concentrations of Cr^{VI} caused oxidative stress in *A. dubius.* As Cr^{VI} concentrations increased, SOD activity decreased progressively, where as the activity of catalase increased in higher concentrations. This result was in agreement with the trend observed in *Carthamus tinctorius* L. in respose to cadmium toxicity[11]. The increase in antioxidant enzyme activity in lower concentrations of Cr^{VI} in *A. dubius* may be attributed to the increased expression of the genes encoding antioxidant enzymes in response to increased production of reactive oxygen species[12]. The decline in antioxidant enzyme activity at higher Cr^{VI} concentration(s) may result from the inactivation of the enzyme by H2O2, which is produced in different cellular compartments or from a number of non-enzymatic and enzymatic processes in cells[13].

Inference in the present study suggest, reactive oxygen species (ROS) could be induced by phytotoxic concentrations of Cr^{VI} leading to increased CAT activities which play a crucial role in detoxification of elevated concentrations of Cr^{VI} possibly via lignifications and physical barrier formation[14]. The declining activity of antioxidant enzymes at higher concentration(s) might be the result of the acute toxic effect which resulted from lipid peroxidation and cell damage, as reflected by the drastic reduction in vigor when compared with control[15].

Table 3: Effects of different concentrations of hexavalent chromium (Cr^{VI}) on SOD and Catalase activity of *Amaranthus dubius* (CO1)

Treatment chromium (mg/kg soil)	Average SOD activity (U/mg)	Average CAT activity (U/mg)
1mg	1.021±0.006	0.02±0.005
10mg	0.996±0.009	0.04±0.005
30mg	0.892±0.014	0.13±0.016
50mg	0.488±0.019	0.15±0.016
70mg	0.456±0.018	0.16±0.024
Control	1.034±0.007	0.02±0

Each mean data with SD presented in the table is an average of 4 replications

Conclusion

The loss of structural integrity and cellular damages in stems and roots of *Amaranthus dubius* treated with hexavalent chromium (Cr^{VI}) clearly indicate that Cr^{VI} has profound negative impact on the physiology, metabolism and growth of the plant. The investigation concludes that Cr^{VI} is toxic to *A. dubius* and degree of toxicity is concentration dependant. The Cr^{VI} toxicity induced changes in anatomy, physiology and morphology and the same is observed to increase as the level of Cr^{VI} concentration increased. Omosun *et* **al.[16],** (2008) have suggested the use of changes in the anatomy of a plant due to a pollution effect as a possible phytoindicator of a polluted environment and therefore the visible anatomical and morphological changes observed in *A. dubius* plants in the present study in response to hexavalent chromium stress can be employed as a phytomonitoring technique for heavy metal Cr^{VI} pollution. Result of the present study also indicates that chromium toxicity caused variations in the production and activity of antioxidant enzymes compared to healthy control seedlings, which clearly indicates that the growth of *A. dubius* has been unfavourably affected by the stress due to chromium toxicity.

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06/06/2023

CERTIFICATE

This is to certify that the corrections/suggestions recommended by the adjudicators have been incorporated in the Ph.D. thesis of Smt. SIMI. R entitled "Chromium (VI) Uptake and Tolerance Potential in *Amaranthus dubius* Mart. ex Thell.: Effect on Genotoxicity, Cell structure, Antioxidative activity, Mineral Nutrition, Growth and Nutritional Value". The contents in the thesis and the soft copy are one and the same.

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