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# A POSSIBILITY OF USING WATERLILY (*NYMPHAEA ALBA* L.) FOR REDUCING THE TOXIC EFFECTS OF CHROMIUM (Cr) IN INDUSTRIAL WASTEWATER

AISHA SALEEM KHAN[*1], MIAN WAJAHAT HUSSAIN[2] AND KAUSER ABDULLA MALIK[3]

[1]*Department of Biological Sciences, Forman Christian College Lahore, Pakistan*
[2]*Department of Biological Sciences, Forman Christian College Lahore, Pakistan*
[3]*Department of Biological Sciences, Forman Christian College Lahore, Pakistan*
[*]*Corresponding author's email: aishasaleemkhan@fccollege.edu.pk*

**Abstract**

This research work reports the potential of waterlily (*Nymphaea alba* L.) a hydrophyte, to accumulate heavy metals like Cr (III) in its roots, shoot and leaves without showing prominent visible symptoms of metal toxicity. Effluent was collected from Ravi which is one of the highly polluted river as compared to other rivers in Pakistan. One of the major reason is industrial wastewater, which is disposed untreated in the Ravi which is a major threat for aquatic life and toxic for human health. It further reports that the water of Ravi river is contaminated with heavy metals like Cd, Cr, Pb, Hg and Zn because their uptake by waterlily plants is an indication that these metals are responsible for polluting Ravi water and this issue need to be resolved on priority basis. In order to estimate the amount of heavy metals in waterlily, different tissues were processed for atomic absorption spectrometry (AAS) which revealed that Cd and Cr (later being in high concentration) were significantly accumulated by waterlily roots, stem and leaves, however, roots were more responsive as compared with aerial parts. Other metals were accumulated in negligible amount in roots, and almost no uptake was reported by stems and leaves. Various anatomical features were used as a tool to support the hypothesis of using waterlily as a hyperaccumulator of Cr in phytoremediation. Reduction fresh and dry weight of root and stem, decrease in chlorophyll content of leaves, inhibition in the cortical and vascular region of stem, and an increase in astrosclereids (supporting parenchyma) formation indicated that Cr was translocated to aerial parts. Increase in astrosclereids might be attributed a mechanism of defense or sensitivity of waterlily plants under Cr stress.

**Key words:** Chromium, Hydrophytes, Hyperaccumulators, Industrial wastewater, Waterlily.

## Introduction

Ravi is one of the important river of Punjab plains. However, due to increase in industrialization and urbanization, it is highly polluted with heavy metals. Sewage including industrial wastewater is drained into the Ravi by many pump stations located along the Bund road. This is reported by many researchers (Javed & Hayat, 1995; Javed 2003; Ubaidullah *et al*., 2004; Rauf *et al*., 2009). Many plants are known to accumulate heavy metals in their tissues and therefore they are important in phytoremediation, which is an emerging and low cost technology, aimed at removing of both organic and inorganic toxic compounds from soil, air or water. Plants can be classified into three main groups depending on their ability to transport metals, 1) accumulators plants which can uptake heavy metals in their aerial parts which can later be harvested 2) plants may be indicators which show level of metal concentration in rhizospheric soil 3) or plants may be excluders which restrict uptake of heavy metals into roots. Over 500 species of flowering plants are known for their potential to accumulate heavy metals. They have high genetic capacity to accumulate huge amount of metals within their shoots. They are classified as hyperaccumulators because the amount of metals detected in these plants is 50-100 higher (depending on metal) than in non-accumulating plants (McGrath & Zhao, 2003).

Hyperaccumulators uptake metals at higher rates and translocate them to shoots and leaves. They behave like diffuse samplers, accumulating pollutants to a higher concentration than their surroundings. However, if plants do not accumulate these metals into their aerial parts, they are not hyperaccumulators and therefore not effective in phytoremediation. *Arabidopsis halleri* and *Thalapsi caerulescens* are model plants which are being used to resolve issues related to phytoremediation as reported by many researchers (Yang *et al*., 2005; Basic *et al*., 2006; Willems *et al*., 2007; Memon & Schroeder, 2009; Verbruggen *et al*., 2009).

Heavy metals are toxic for plant growth and interfere with many biochemical pathways which are crucial for their developmental stages. Main reasons for heavy metals entry in environment include increase in industrialization, mining activities, common use of insecticides and pesticides, mismanagement of waste disposal and traffic smoke etc. Many heavy metals like Cd, Cr, Cu, Hg, Pb, Ni and Zn cause inhibition in seed germination (Khattak *et al*., 2015; Li *et al*., 2005; Soudek *et al*., 2010) root and shoot elongation, reduction in vascular tissues, damage to photosynthetic pigments (Singh *et al*., 1996; Baek *et al*., 2012 and Strzalka *et al*., 2013) and delay in floral initiation (Chaudhry & Khan, 2006), an increase in crystal formation in some plants (Khan & Rehan, 2014) and also affect the proteomics causing oxidative stress (Garcia *et al*., 2006).

Chromium is another serious pollutant which is toxic for plant growth and development, however toxicity of Cr depends upon its valence state. Cr (III) is mobile in plant tissues and taken up by aerial parts where it interferes with rate of photosynthesis by affecting photosynthetic pigments (Shanker *et al*., 2005). There is a progressive increase in Cr uptake in roots of many plants like *Nelumbo nucifera* (Vajpayee *et al*., 1999), *Helianthus annuus*, *Vicia faba* and *Zea mays* (Kocik & Ilavsky, 1994). In higher plants Cr toxicity is reported at 100 μM. $kg^{-1}$ dry weight (Davies *et al*., 2001). Cr at concentration of 20ppm and 80ppm can cause 32-57% reduction in growth rate of sugarcane bud (Jain *et al*., 2000). However a significant reduction in dry biomass is reported at 200 or 400 mg/kg at flowering stage of *Sinapsis alba* (Hanus & Tomas, 1993).

## Materials and Methods

In order to explore the role of waterlily as hyperaccumulator, many morpho-anatomical features were studied and compared with plants growing in pond water (which were taken as control). Fresh and dry weight analysis of root, stem and leaves and chlorophyll estimation through spectrophotometry was done for both control and experimental samples. All values were calculated and were compared with control plants. Anatomical sections of plants sections were prepared through rotary microtome in order to evaluate the structural changes in plants placed in Ravi water. Atomic absorption spectrometry was done for plants for estimation of Cr accumulated in plant tissues, in order to observe whether waterlily can be used in phytoremediation.

Following methodologies were followed in order to meet the project objectives.

**i. Sample collection:** Waterlily plants were placed in tubs filled with water collected from Ravi, however, five tubs were with tap water which served as control. The samples were collected from the surface drains with the help of an open bucket connected with a rope and then transferred into air tight containers. Five replicates were selected for each sample. These tubs were kept in experimental area of Forman Christian College, Botanic Garden.

**ii. Measurement of fresh and dry weight:** Roots, stem and leaves from control and Ravi water were rinsed and then fresh weight was weighed. Samples were dried in oven at 75 -77°C for 16 h and then weighed again. Difference in fresh and dry weight of control and treated plants is shown in figure 1.

**iii. Chlorophyll estimation:** Chlorophyll is soluble in acetone when the sample is macerated in acetone (Arnon 1949). Optical density of the extract was measured at 645nm and 663nm. Leaves of waterlily were weighed ($3cm^2$) and were cut into small pieces. They were crushed in mortar with 5 ml of 80% acetone for 4 to 5 minutes until the paste was prepared. Rinsing was done using 1 ml of 80% acetone. Acetone was added to make final volume upto 10 ml total in test tube. Then, a sample measuring 3ml was added from the top of the sample into the tube for spectrometry. First the wavelength was set at 663λ and the readings for 80% acetone were made as zero. Then the absorbance of sample was recorded in spectrophotometry. Values of chlorophyll content in control and treated samples are shown in Table 1.

**Table 1. A comparison of chlorophyll content in control (pond water) and treated waterlily (*N. alba*) plants placed in Ravi water.**

| Samples | Chlorophyll 'a' (mg/g) | Chlorophyll 'b' (mg/g) | Total chlorophyll (mg/g) |
|---|---|---|---|
| Pond water | 0.72 | 1.2 | 1.93 |
| Ravi water | 0.67 | 1.11 | 1.78 |

**iv. Anatomical techniques:** In order to observe anatomical changes, Sanderson (1994) method was used; Plants were cut into small size pieces with a razor blade and were fixed immediately in Corney's modified fluid. After fixation, plant specimens were dehydrated with gradual increase of ethanol in order to make tissues clear. After dehydration samples were infiltrated with granular paraffin wax (Merck). It was carried out in an embedding oven, at 55°C to 60°C by giving several washes with paraffin wax, until the wax became transparent. After infiltration, the specimens were embedded in an embedding block and paraffin wax was slightly trimmed around the section. This was followed by fixing tissues on the wooden blocks. Sections were cut with the help of a rotary microtome and ribbon was placed in a warm water tray and then affixed to the clean slides with adhesive comprising of 50% egg albumin and 50% glycerine. Staining was done in toluidine blue stain. Canada balsam was used to coat slides permanently.

**v. Atomic absorption spectroscopy:** Plant material was weighed upto 0.10 g in a mixture of 25 ml of concentrated nitric acid and 10 ml of concentrated sulphuric acid was digested in a beaker under a fume hood (Soylak *et al*., 2004). Sample was heated (with watch glass over the beaker) and mixture was added slowly to ~50 ml of distilled water in a 100 ml volumetric flask. Beaker was rinsed several times to ensure that all material is transferred and was diluted to the mark with distilled water. All readings were recorded through atomic absorption spectrometer (Model 210 VGP).

## Results and Discussion:

Current research work reported that Ravi is polluted with toxic metals like Cd, Cr, Co, and Zn because waterlily plants placed in water from Ravi showed Cr accumulation in significantly high concentration (measured by AAS) in roots which was further verified by reduction in cortical and vascular parenchyma of root and stem (Figs. 1-10).

Waterlily plants from Ravi effluents showed reduction in chlorophyll content, inhibition of cortical aerenchyma and vascular tissues of stem, suggesting that Cr was translocated to aerial parts (Figs. 3 & 5). Cr uptake resulted in decrease in fresh and dry weight as well as in reduction of chlorophyll content of leaves (Fig. 1 & Table 1). Similar finding are reported by many researchers (Iqbal *et al*., 2001; Vajpayee *et al*., 2000; Mei *et al*., 2002). Decrease in height of plants (Hanus and Tomas, 1993; Joseph *et al*., 1995 and Barton *et al*., 2000) and leaf area (Karunyal *et al*., 1994; Jain *et al*., 2000) under Cr stress is one of the well-documented effects of Cr.

Cr uptake by aerial parts caused inhibition in chlorophyll synthesis which further supports AAS results, indicating that Cr was translocated to leaves and stem. Chlorophyll estimation showed that Chl 'a' showed more sensitivity than Chl 'b' in *N. alba* which affects the rate of photosynthesis (Vajpayee *et al*., 2000). Many workers have reported that Cr uptake is related with inhibition in rate of photosynthesis as it interferes with chlorophyll biosynthesis (Hayat *et al*., 2012), functioning of PSI and PSII (Shanker *et al*., 2005). Cr also causes disorganization of chloroplast ultrastructure and inhibition of electron transport process including diversion of electrons from PSI to Cr. Inhibition in leaf area index and chlorophyll biosynthesis in waterlily roots might be due to interference of Cr with PSI and thereby reducing the rate of photosynthesis. Cr uptake is an active mechanism which involves carriers of essential anions like sulphates (Cerventes *et al*., 2001). Immobilization of Cr (VI) is also mostly reported in less toxic form, in vacuoles of root cells (Shanker *et al*., 2003) and therefore it is less toxic for plants. However Cr (III) and in some cases Cr (VI) cross the root endodermis through symplast (Shanker *et al*., 2005).

Amount of Cr accumulated in roots of waterlily was significantly high after 30 days as compared with control (Fig. 3) which suggests that it can be used in removing Cr from industrial waste water and its potential in phytoremediation can be explored further through characterization of genes which are involved in Cr translocation. Metal uptake by plants involves specific proteins and a number of metals transporters are located on plant membranes like plasma membrane transporters, tonoplast transporters, transporters for xylem loading and many endomembrane transporters. Further work can reveal about channels and proteins involved in metal transport in waterlily plants. Some families are identified in *A. thaliana* like ZIP and HMA (heavy metal ATPase). ATNramp3 is one of the metal ion transporter of vacuolar membrane expressed in *A. halleri*, that is involved in complementing deficiencies in Fe and Mn (Filatov *et al*., 2007). TcHMA4, another channel protein, plays an important role in translocation of heavy metals transport from root to shoot (Milner & Kochian, 2008). Techniques like cryo-electron microscopy and x-ray crystallography can provide us with the information of translocators involved in heavy metals translocation in these plants to further explore their role in phytoremediation.

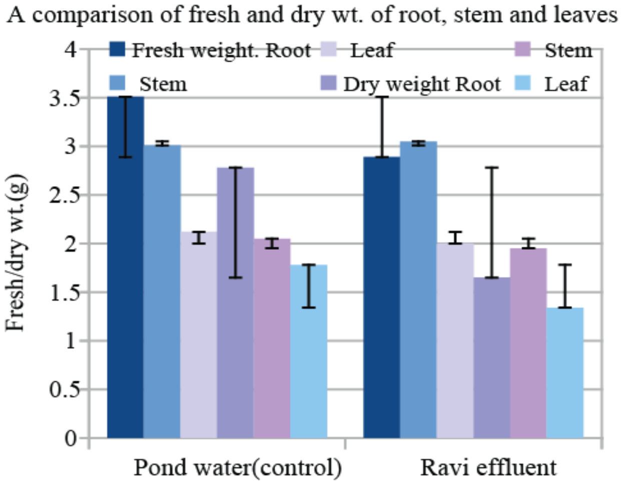


Fig. 1. Fresh and dry weight (g) of root, stem and leaf in control and treated waterlily (*N. alba*) plants.

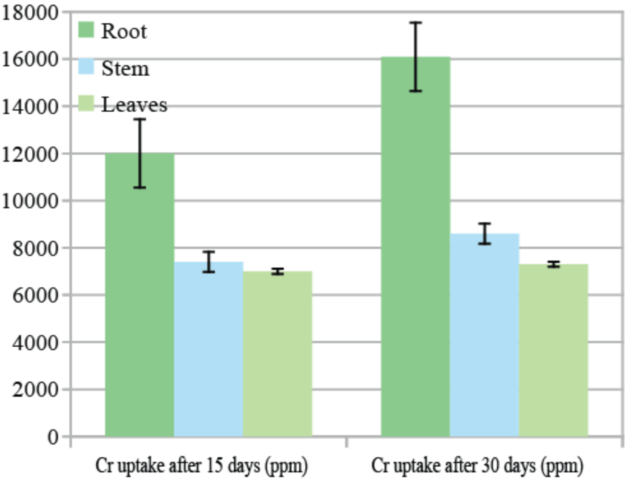


Fig. 2. Cr uptake in waterlily (roots, stem and leaves) placed in Ravi water after 15 and 30 days

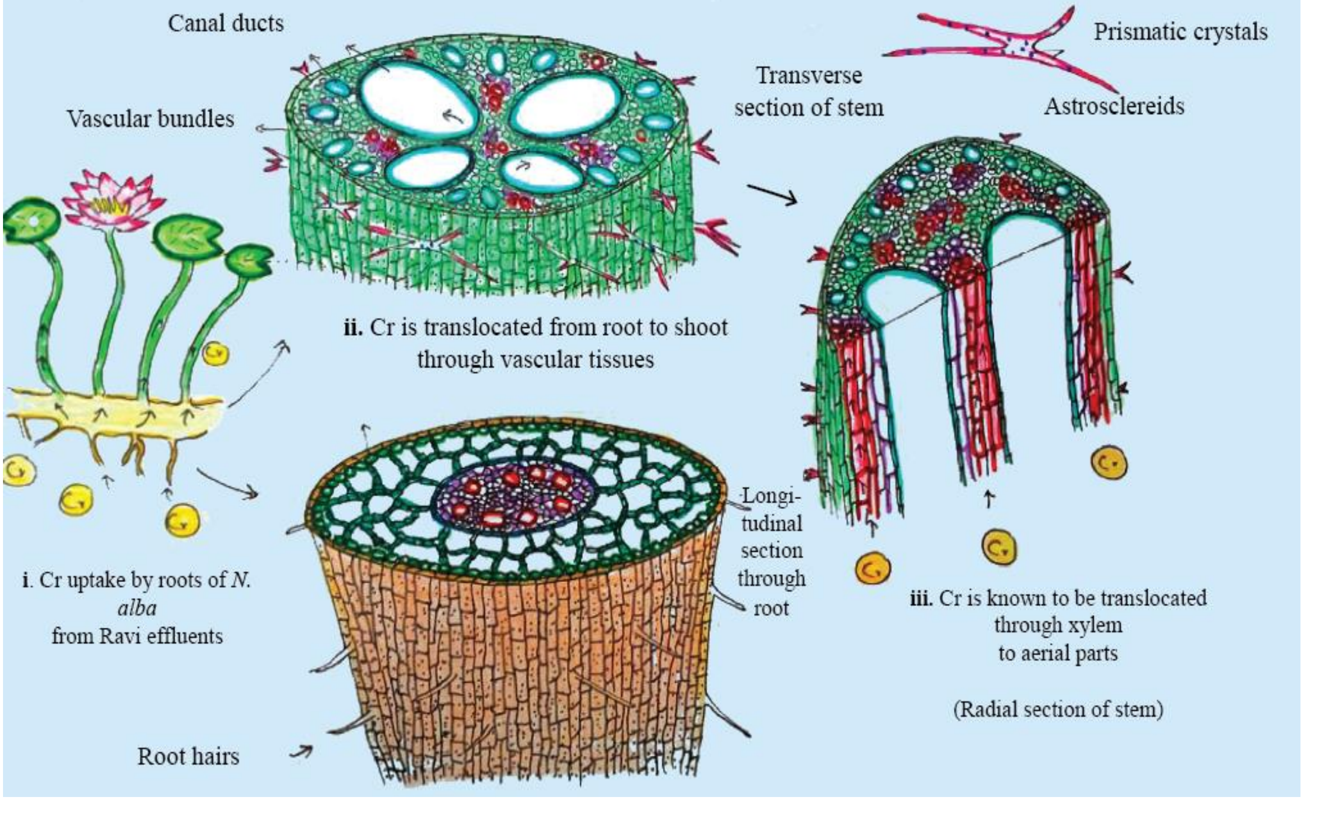


Fig. 3. A hypothetical explanation of Cr uptake, translocation and interference with astrosclereids formation in waterlily grown in Ravi water.

In current work, reduction in height and vascular tissues and less chlorophyll content might be due to oxidative damage induced by Cr translocation through effluent collected from Ravi. However, many plants have developed defensive mechanisms under metals stress which resist the their toxic effects by making them either immobilized and detoxify them by sequestrating in vacuoles (Seregin *et al*., 2004). Several non-specific defense systems involved may include changes in chemical composition of cell wall (callose and suberin deposition), synthesis of osmolytes (proline) and polyamines and changes in hormonal balance (Cho *et al*., 2003). Cr toxicity is attributed to oxidative damage and disturbance in water status, which allows a large amount of Cr (VI) to enter roots passively, translocating it to aerial parts, leading to oxidative damage of photosynthesis including mitochondria and thereby affecting the overall efficiency of plant metabolism (Shanker *et al*., 2005).

Cr uptake in waterlily plants caused an increase in number of astrosclereids (star-shaped sclerenchyma) in leaves and in stem (Figs. 5-8). There was some reduction in cortical aerenchmya of roots and stem, however, Cr uptake did not significantly interfered with the vascular growth as there was negligible inhibition. Astrosclereids is a supporting sclerenchyma which provides support and protection to many plants. An increase in number of these cells, show the defensive mechanism of waterlily plants under stress conditions. Since astrosclereids are lignified parenchyma cells, their high number is an indication that Cr uptake also interferes with shikimic acid pathway (Oberg *et al*., 2007) which is mainly responsible for lignin formation in plants, however, it need to be studied further.

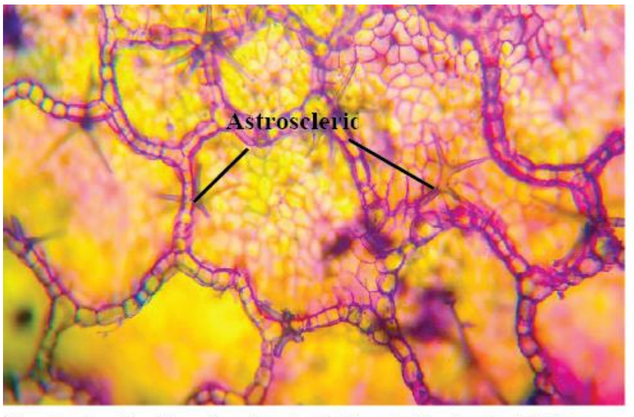

Fig. 4 . Leaf epidermis of control of waterlily (under 10X)

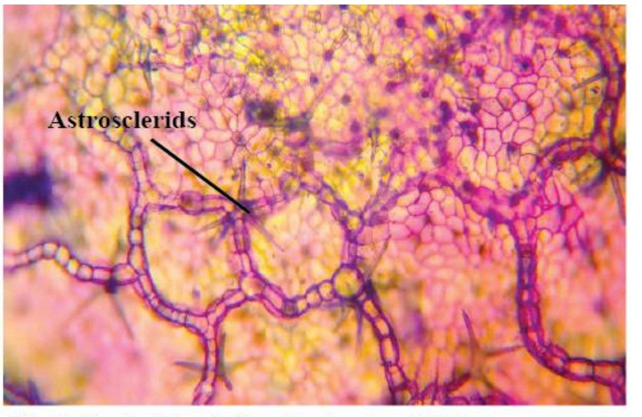

Fig. 5. Leaf epidermis from Ravi water at 10X.

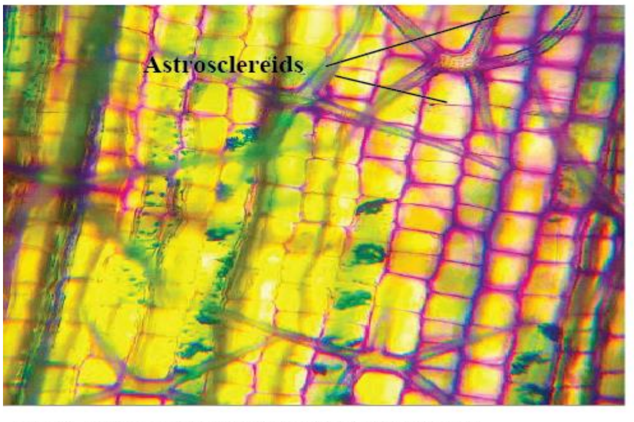

Fig. 6. Astrosclereids in stem of waterlily (10X) .

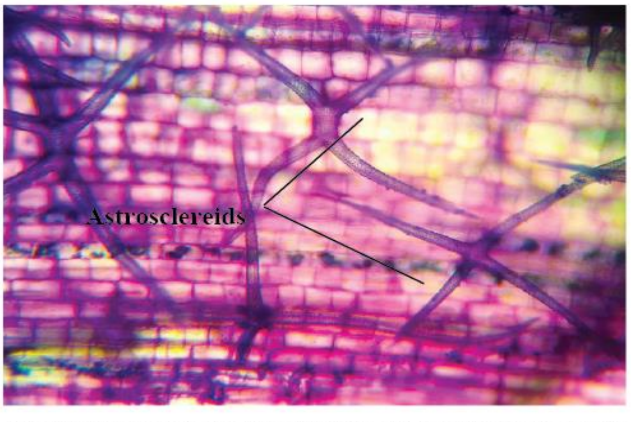

Fig. 7 Astrosclereids in stem placed in effluent from Ravi (10X).

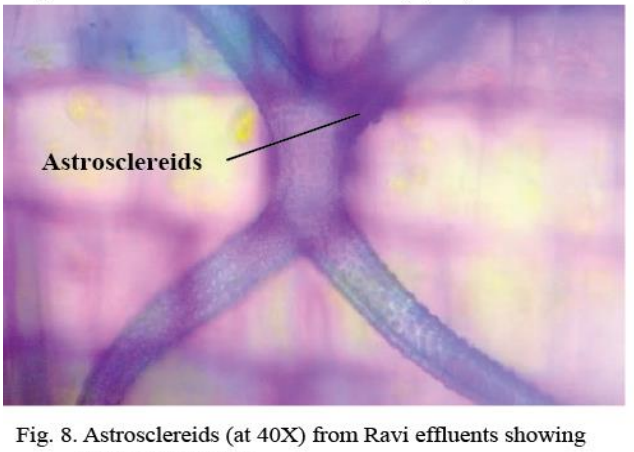

Fig. 8. Astrosclereids (at 40X) from Ravi effluents showing prismatic crystals

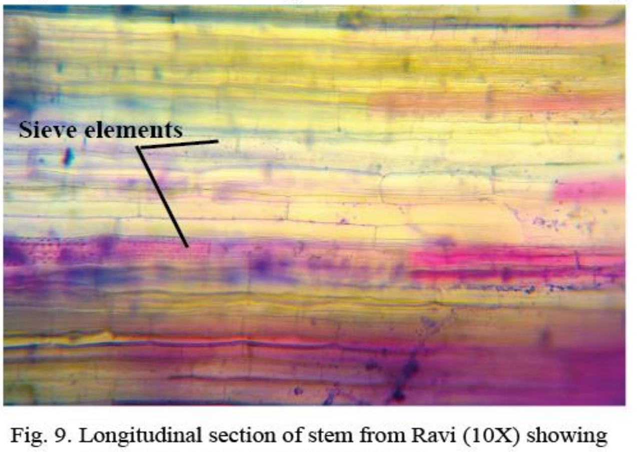

Fig. 9. Longitudinal section of stem from Ravi (10X) showing reduced cortical and vascular tissue

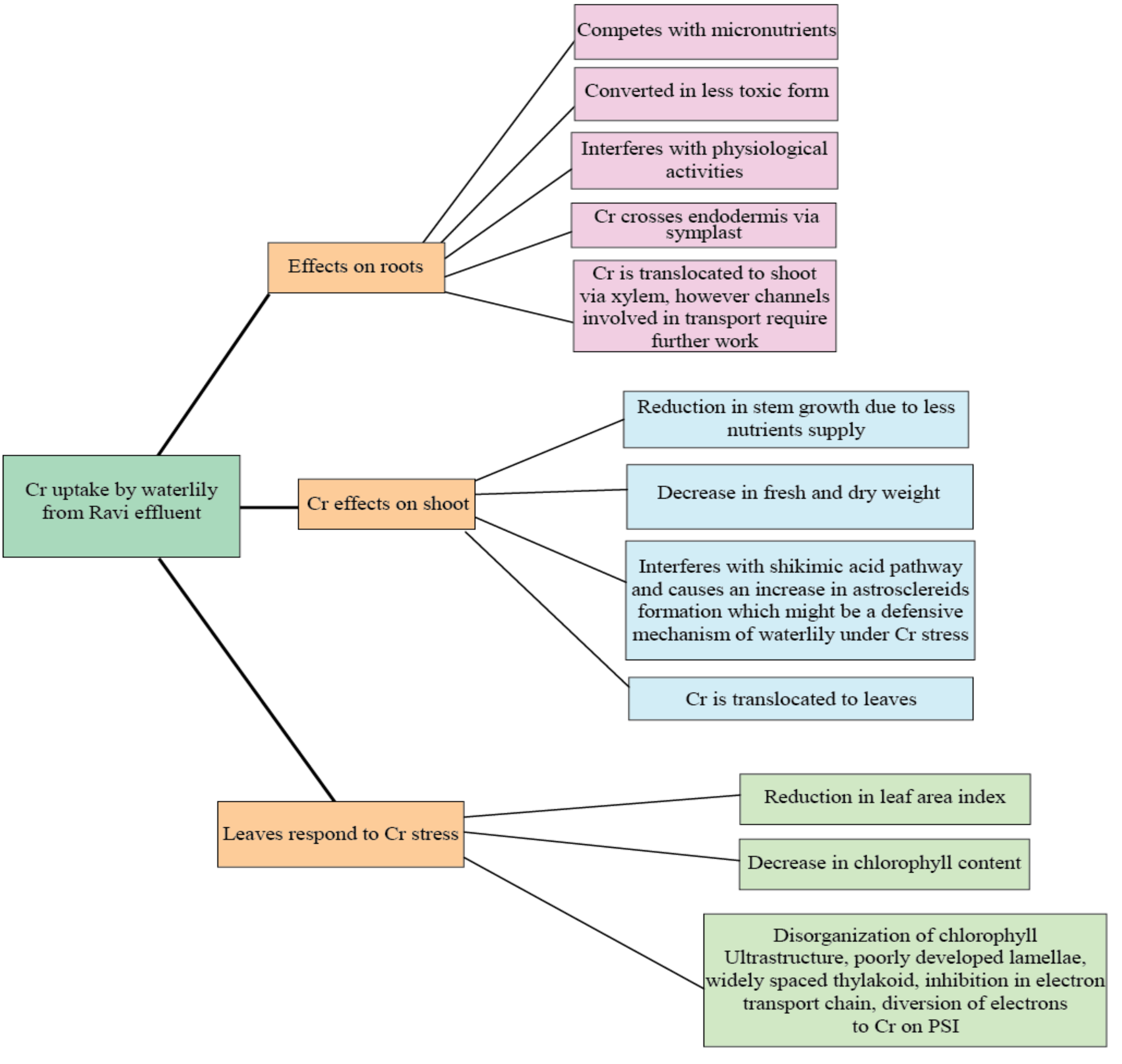


Fig. 10. A hypothetical explanation of Cr metabolism in *N. alba*.

## Conclusion and future perspectives

Accumulation of Cr in waterlily plants grown in Ravi water suggests its possible role in phytoremediation. Anatomical research further supports this hypothesis as decrease in cortical and vascular parenchmya and increase in number of astrosclereids (supporting sclerenchyma) in their stem and leaves, indicates defensive mechanisms of waterlily plants under Cr tress. However, role of Cr in phytoremediation can be further explored through (1) electron microscopy and x-ray diffraction can reveal transporters of heavy metals involved in translocation of Cr in waterlily plants (2) or through analyzing metabolic pathways which are responsible for the formation of supporting parenchmya (astrosclereids) under Cr stress.

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