CHAPTER III: MATERIALS AND METHODS
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A. CULTURE OF PLANTS

Culture of plants and experimental set up were done by adopting the method of Göthberg et al. (2004). *I. aquatica* plants were collected from unpolluted water bodies in and around Irongmara, Silchar, Assam, and grown in soil flooded with 50% Hoagland nutrient solution (HS) having pH 5.8 - 6.2 (Fig 1a). *I. aquatica* has been used as test organism in this study because it can be easily propagated and is perennial in nature. Besides this it occurs wild on moist soil or mud along the margins of slow-flowing streams, fresh water ponds, ditches, marshes and wet rice fields (Prasad et al., 2008) thus having a wide variety of habitat. This plant is also consumed as a leafy vegetable throughout the northern and northeastern parts of India as well as in southeast Asia.

Hoagland nutrient solution (50%) contained 1ml of 1M Mg SO$_4$7H$_2$O, 2.5 ml of 1M Ca(NO$_3$)$_2$4H$_2$O, 0.5ml of 1M KH$_2$PO$_4$, 2.5 ml of 1M KNO$_3$, 0.5 ml micronutrient stock (2.86 g of H$_3$BO$_3$, 0.22g of ZnSO$_4$4H$_2$O, 1.81g of MnCl$_2$4H$_2$O , 0.08g of CuSO$_4$5H$_2$O and 0.025g of Na$_2$MoO$_4$2H$_2$O in 1L of H$_2$O) and 5 ml of 0.01M Fe-EDTA per liter of distilled water (Hoagland and Arnon, 1950).

The soil and nutrient medium were renewed every month and the water level was maintained by adding dechlorinated tap water. One week before the beginning of metal exposure experiments, 20-25cm long shoots were cut and placed in 50% HS in order to acclimatize them for a period of 7 days in laboratory condition and also to produce new roots.

The test cultures were maintained at an ambient temperature range of 23.4 ± 2.8°C in test chambers provided with light intensity of 100 - 120 μmol m$^{-2}$ s$^{-1}$ around the culture vessels following a photoperiod of 12h. Fig 1b shows the plants during the exposure period.
Figure 1a: Culture of *Ipomoea aquatica* Forsk. before exposure.

Figure 1b: *I. aquatica* under exposure condition.
B. CHEMICALS

The present study tested the effects of two heavy metals, viz., copper (Cu) and nickel (Ni) on *I. aquatica*. Cu as Copper sulfate (CuSO$_4$.5H$_2$O) was added to the 50% nutrient medium to obtain different graded concentrations of Cu for performing acute and chronic toxicity tests. The other heavy metal, Ni was added as Nickel sulfate (NiSO$_4$.6H$_2$O) at different graded concentrations for acute and chronic toxicity tests. 50% HS medium without added Cu and Ni served as control medium for plant growth.

C. ACUTE TOXICITY TESTS

After the acclimatization period, plants were exposed to graded concentrations (0.25, 2.54, 4.58, 8.14, 14.25 and 25.45 mg L$^{-1}$) of Cu and Ni (0.22, 2.23, 4.02, 7.15, 12.51 and 22.37 mg L$^{-1}$) for a time period of 15 day (Sharma *et al.*, 2011; Mishra *et al.*, 2014). Laboratory acclimatized plants without added Cu and Ni served as control. All the Cu and Ni concentrations used in the present study represented actual and not nominal concentrations of the two elements. In each Cu treatment 3 replicates were used along with 3 control plants and the experiment was repeated 3 times. In the experiment with Ni, 3 replicates were used in each treatment along with 3 replicates under control treatment and the experiment was repeated 2 times. In this test, toxicological endpoints such as changes in shoot height (SH), number of new nodes (NN), number of new leaf (NL) and number of dead plants (DP) with respect to those recorded at the beginning of the experiment (0 day) were observed on 3$^{rd}$, 6$^{th}$, 9$^{th}$, 12$^{th}$ and 15$^{th}$ day of the experiment. SH were measured using thread and a well marked meter scale. During the acute toxicity tests, the test solution was changed every week.
D. CHRONIC TOXICITY TESTS

The chronic toxicity tests were conducted with an exposure period of 30 days (Azooz et al., 2012; Mishra et al., 2014). The acclimatized plants were exposed to 0.025, 0.08, 0.13, 0.25, 2.54 and 4.58 mg L\(^{-1}\) of Cu and to 0.02, 0.11, 0.22, 2.23, 4.02 and 7.14 mg L\(^{-1}\) of Ni. These concentrations were actual elemental and not nominal concentrations. The concentrations of Cu and Ni used in the chronic study were selected on the basis of the results obtained in the acute toxicity tests. Twenty one test plants were individually exposed to each concentration of the two heavy metals. A control set consisting of twenty one plants were also similarly maintained in the nutrient medium without added Cu or Ni. The test solution during these tests was renewed every week.

I. Measurement of growth

The changes in shoot height (SH) were measured using a meter scale and thread. The changes in the number of new nodes (NN), number of new leaf (NL), number of new tiller (TN), width of leaf blade (WLB) with respect to those recorded at the beginning of the experiment (0 day) were measured on 3\(^{rd}\), 6\(^{th}\), 9\(^{th}\), 12\(^{th}\), 15\(^{th}\), 18\(^{th}\), 21\(^{st}\), 24\(^{th}\), 27\(^{th}\) and 30\(^{th}\) day of the exposure. In addition to this, changes in fresh weight (FW) were also observed at the end of the experiment on 30\(^{th}\) day of the exposure. Extreme care was taken while taking the measurements to ensure that the plants were not subjected to any kind of stress. While measuring FW, the plants were carefully wiped with clean tissue paper to avoid fluctuation in the weights.

II. Chlorophyll content

Chlorophyll content was measured spectrophotometrically (Arnon, 1949). On 3\(^{rd}\), 9\(^{th}\), 18\(^{th}\) and 30\(^{th}\) day of the exposure, leaves from both control and metal exposed plants were taken to
compare the chlorophyll content. However, in the case of 7.14 mg L$^{-1}$ Ni exposed plants, chlorophyll content was measured till the 18$^{th}$ day, since the exposed plants were devoid of leaves after this period. 100 mg of small cut pieces of fresh leaves were ground in a clean mortar with the help of a pestle. 4 - 5 ml of 80% acetone was added and the tissues were ground to a fine pulp. The extract was carefully filtered through Whatman no.1 filter paper. If the pellet was greenish in colour it was recrushed with 4 - 5 ml of 80% acetone and filtered again. Then the extract was centrifuged at 2500 rpm for 30 min. The supernatant so obtained was made upto 10ml by adding 80% acetone. From the extract so obtained the absorbance was measured spectrophotometrically at two different wavelengths, viz. 645 and 663 nm.

Calculation:

\[
\text{mg Chl } a/\text{g tissue} = [12.7(D_{663}) - 2.69(D_{645})] \times \left[\frac{V}{1000} \times W\right]
\]
\[
\text{mg Chl } b/\text{g tissue} = [22.9((D_{645})) - 4.68(D_{663})] \times \left[\frac{V}{1000} \times W\right]
\]
\[
\text{mg total Chl/g tissue} = [20.2(D_{645}) + 8.02(D_{663})] \times \left[\frac{V}{1000} \times W\right]
\]

Where, \( V \) = Final volume of the extract (ml).
\( W \) = Weight of the sample in gram.
Chl \( a \) = Chlorophyll \( a \).
Chl \( b \) = Chlorophyll \( b \).
\( D_{645} \) = Optical density at 645 nm.
\( D_{663} \) = Optical density at 663 nm.

**III. Protein content**

Reagents required:

a) Reagent A: 2% sodium carbonate in 0.1 N sodium hydroxide.
b) Reagent B: 0.5% copper sulphate in 1% potassium sodium tartarate.
c) Reagent C: Alkaline copper solution prepared by mixing 50 ml of A and 1 ml of B fresh before use.

d) Reagent D: Folin Ciocalteau Reagent.

e) Reagent E: 0.1 M sodium phosphate buffer solution (pH 7).

Procedure:

Protein estimation of the stem and leaf tissues were done using the method of Lowry et al. (1951) on 3\textsuperscript{rd}, 9\textsuperscript{th}, 18\textsuperscript{th} and 30\textsuperscript{th} day of the exposure in 0.025 - 0.25 mg L\textsuperscript{-1} Cu and 0.02 - 7.14 mg L\textsuperscript{-1} Ni exposed plants. In the case of plants exposed to 2.54 and 4.58 mg L\textsuperscript{-1} Cu, protein estimation of the stem and leaf samples were done only upto day 9 and 3, respectively, due to the occurrence of necrosis in the stem tissues and drying up of leaf. 100 mg of fresh leaf and stem were weighed and ground well with a mortar and pestle in 5 ml of the phosphate buffer. To a known volume of the extract sample drawn from the supernatant collected after centrifugation, 5ml of reagent C were added and mixed well and then allowed to stand for 10 - 15 minutes. This was followed by the addition of 0.5 ml of reagent D and the resultant solution was left to stand for 30 minutes. Blue colour was developed and the absorbance was measured at 650nm in a spectrophotometer. The prepared solution devoid of sample was used as the blank. The concentration of protein was determined using standard bovine serum albumin (BSA) solution. Fig 2a shows the standard curve of protein prepared by taking 0.1 – 1 ml of the BSA solution (5 mg ml\textsuperscript{-1} diluted 20 times).

IV. Total carbohydrates content

Reagents required:

a) 80% ethanol, b) 95% sulphuric acid, c) 0.2% anthrone reagent
Procedure:

Total carbohydrates content in the stem and leaf tissues were estimated using the method of Dubois et al. (1956) on 3rd, 9th, 18th and 30th day of the exposure in 0.025 - 0.25 mg L\(^{-1}\) Cu and 0.02 - 7.14 mg L\(^{-1}\) Ni exposed plants. In the case of plants exposed to 2.54 and 4.58 mg L\(^{-1}\) Cu, carbohydrates estimation of the stem and leaf samples were done only up to day 18 and 9, respectively because of the appearance of necrosis in stem tissues due to which leaf were all fallen. 100 mg of fresh leaf and stem was extracted with 80% ethanol (1:10 w/v). The extract was then made aqueous with a known volume of distilled water (1 ml). A known volume of the extract was heated with 4 ml of anthrone reagent prepared in concentrated sulphuric acid for 8 minutes in a boiling water bath, cooled rapidly. The absorbance of the resultant solution was measured spectrophotometrically at 620 nm. The prepared solution devoid of sample was used as the blank. The concentration of the carbohydrates was determined using standard glucose solution. Fig 2b shows the standard curve of carbohydrates prepared by taking 2 - 10 mg ml\(^{-1}\) of glucose.

V. Ascorbic acid content

Reagents required:

a) 4N H\(_2\)SO\(_4\)

b) Iodine (I\(_2\)) solution: 6.35 g of Iodine in 12.5 g of Potassium iodide dissolved in 1 litre of distilled water.

c) N/20 Sodium thiosulphate (Na\(_2\)S\(_2\)O\(_3\)) solution

d) Starch solution: Starch solution is prepared by dissolving starch in warm distilled water in the ratio of 1:100 w/v. The solution is heated till it becomes clear.
Figure 2a: Standard curve of protein.

Figure 2b: Standard curve of carbohydrates.
Ascorbic acid content was measured by using the method of Bailey (1974) on 3rd, 9th, 18th and 30th day of the exposure in 0.025 - 0.25 mg L⁻¹ Cu and 0.02 - 7.14 mg L⁻¹ Ni exposed plants. In the case of plants exposed to 2.54 and 4.58 mg L⁻¹ Cu, ascorbic acid estimation of the stem and leaf sample was done only upto day 9 and 3 respectively due to the occurrence of necrosis in the stem tissues and devoid of leaf. Plant extract was prepared by taking fresh leaf and stem with distilled water in the ratio 1:10 w/v. 10 ml of the centrifuged sample extract was pipetted out in a conical flask and 10 ml of distilled water was added, followed by 1 ml of 4N H₂SO₄. To this, 20 ml of iodine solution was added and allowed to stand for 1 - 2 minutes. This resultant solution was titrated with sodium thiosulphate solution of known strength till dark brown colour faded to light brown or pale yellow. 1 – 2 ml of the starch solution was added till dark blue colour appeared which was further titrated till the blue colour was discharged with one drop and burette reading was noted.

Calculation:

Standardization of Na₂S₂O₃ and I₂ solutions

\[
\text{Strength of Na}_2\text{S}_2\text{O}_3 \text{ solution (S)} = \frac{\text{Strength of } K_2\text{Cr}_2\text{O}_7 \times \text{Volume of } K_2\text{Cr}_2\text{O}_7}{\text{Volume of } Na_2\text{S}_2\text{O}_3}
\]

\[
\text{Strength of I}_2 \text{ solution (S1)} = \frac{\text{Strength of } Na_2\text{S}_2\text{O}_3 \times \text{Volume of } Na_2\text{S}_2\text{O}_3}{\text{Volume of I}_2}
\]

If 10 ml of I₂ solution = x ml

So, 20 ml of I₂ solution = 2x

If Vit C + 20 ml of I₂ consumed = y ml

So, Vit C consumed = y – 2x ml

We have, 1 ml of 1 N Na₂S₂O₃ solution = 0.08806 g of Vit C
i.e. 1 ml of $S = 0.08806xS$ g

Now, $y - 2x$ of $S = 0.08806 \times S \times (y - 2x)$ g of Vit C

**VI. Enzyme activity**

1. **Catalase (CAT)**

   CAT activity on stem and leaf tissues of the Cu exposed plants at 0.025 - 0.25 mg L$^{-1}$ and Ni exposed plants at 0.02 - 7.14 mg L$^{-1}$ were analyzed on 3rd, 9th, 18th and 30th day of the exposure. In the case of plants exposed to 2.54 and 4.58 mg L$^{-1}$ Cu, stem and leaf tissues were analyzed only up to 9 and 3 day of the exposure, respectively due to the occurrence of necrosis in the stem tissues and drying up of leaf.

Reagents required

a) Potassium phosphate buffer solution (PBS) (50 mM)

b) Hydrogen peroxide ($H_2O_2$) 30 mM: 0.34 ml of 30% $H_2O_2$ is diluted with phosphate buffer to 100 ml.

Enzyme extract:

0.5 g of fresh leaf or stem were ground with 8 ml solution containing 50 mM potassium phosphate buffer (pH 7.0) and 1% polyvinylpolypyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 30 min at 1 - 4°C and supernatant was collected for enzyme assay (Hakiman and Maziah, 2009).

Procedure:

CAT activity was determined according to the method of Aebi (1984) with some modifications after Karuppanapandian et al. (2009). In this method, 0.2 ml of enzyme extract was taken along with 1.8 ml of PBS (pH 7.0) in a test tube. To this assay mixture 1 ml of 30
mM H₂O₂ was added. The changes in the rate of decomposition of H₂O₂ were measured spectrophotometrically at 240 nm during the reaction.

2. Guaiacol Peroxidase (GPOD)

GPOD activity in stem and leaf tissues of the Cu exposed plants at 0.025 - 0.25 mg L⁻¹ and Ni exposed plants at 0.02 - 7.14 mg L⁻¹ were analyzed on 3rd, 9th, 18th and 30th day of the exposure. In the case of plants exposed to 2.54 and 4.58 mg L⁻¹ Cu, stem and leaf tissues were analyzed only upto 9 and 3 day respectively due to the occurrence of necrosis in the stem tissues and drying up of leaf.

Reagents required:

a) Potassium phosphate buffer 0.1 M (pH 7.0)

b) Guaiacol solution 20 mM

c) Hydrogen peroxide solution 12.3 mM

Enzyme extract:

1 g of fresh leaves and stem was ground with 8 ml of phosphate buffer with the help of mortar and pestle. The homogenate was centrifuged at 10,000 at 4°C for 30 min (Vidyasagar et al., 2009).

Procedure:

Enzyme activity was estimated using the method of Sadasivam and Manickam (1996) with some modifications after Vidyasagar et al. (2009). The 3.0 ml of assay mixture comprised of buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml of hydrogen peroxide solution in a cuvette. Absorbance was measured at 436 nm in a spectrophotometer for every 30 seconds upto 3 minutes. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 m M⁻¹ cm⁻¹).
VII. Scanning Electron microscopy (SEM) studies

Reagents required:

a) 2.5% glutaraldehyde

b) 0.1M sodium cacodylate buffer

c) Graded concentrations of acetone

For SEM studies, stem tissues exposed to 0.025 mg L\(^{-1}\) of Cu and 0.02 and 0.22 mg L\(^{-1}\) of Ni were cut with a new stainless steel razor blade on the 30\(^{th}\) day after the completion of exposure in the chronic toxicity experiment. Stem tissue samples from control plants grown in HS 50% without added Cu and Ni were also collected for comparison. The cut stem tissue samples were fixed in 2.5% glutaraldehyde for 4 hours and kept in 0.1M sodium cacodylate buffer at 4°C for 24 h (Gupta and Gupta, 2004). The samples were then dehydrated in graded concentrations of acetone (30% to 100%) for 15 minutes with 2 changes for each acetone concentration. After dehydration the samples were coated with gold in a fine coat ion sputter JFC 1100 and examined in a scanning electron microscope (JEOL JEM-6360).

VIII. Metal accumulation

Digestion of plant samples for metal estimation was done by adopting the method of Gupta (1996). Stem, root and leaf of the metal exposed plants along with those of control plants were dried to constant weight at 60 ± 2°C, and digested to dryness with concentrated reagent grade nitric acid. The residue was dissolved in distilled water and metal content was determined with a flame atomic absorption spectrophotometer (Perkin Elmer 3110).
1. Copper

An experiment was conducted to understand the pattern of Cu accumulation in different tissues at different durations of exposure in *I. aquatica* plants. For this, nine plants were exposed to 2.54 mg L\(^{-1}\) Cu for a period of 15 days along with the control. Stem, root and leaf of the exposed plants were collected on 5\(^{th}\), 10\(^{th}\) and 15\(^{th}\) day of exposure. Stem, root and leaf of control plants were collected on 15\(^{th}\) day of exposure. The collected tissues were analyzed for estimating Cu concentration.

In a second set of experiment, three plants each were subjected to 0.13, 0.25 and 4.58 mg L\(^{-1}\) of Cu for a period of 30 days. Control comprising of three plants were maintained in HS 50% without added Cu for the same period of time. After day 30 root, stem and leaf tissues were collected from Cu exposed plants and also from control. The collected tissues were digested with nitric acid as described above and were analyzed for Cu concentration.

2. Nickel

Three plants each were subjected to 0.11, 0.22 and 4.02 mg L\(^{-1}\) of Ni for a period of 30 days. Control comprising of three plants were maintained in 50% HS without added Ni for the same period of time. After day 30, root, stem and leaf tissues were collected from Ni exposed plants and from control. The collected tissues were digested separately with nitric acid as described above and were analyzed for Ni concentration.

E. NECROSIS ANALYSIS

Plants exposed to both copper and nickel were monitored for the appearance of necrotic symptoms on their stem. Appearance of slight discoloration producing a brownish or pinkish tinge was taken as the first sign of necrosis and termed as early necrosis (EN). Further necrotic degradation characterized by deeper discoloration was termed as advanced necrosis (AN). Stem
tissue samples were collected and analyzed at different points of time based on the onset and progress of necrosis at different Cu and Ni concentrations.

I. Copper

1. Biochemical analysis

Plants were subjected to 0.025 - 4.58 mg L\(^{-1}\) Cu for a period of 70 days along with control plants. In this experiment eighteen plants were exposed to each concentration of the Cu. Control comprising of eighteen plants were also similarly maintained in HS 50% without added Cu. Some non-necrotic (NN) fragments remaining in plants exposed to 2.54 and 4.58 mg L\(^{-1}\) Cu treatment were placed in HS 50% media without added Cu and observed for 20 days. These samples were termed as recovery (RC). The changes in the length of EN and AN stems were measured using thread and scale. Both EN and AN as well as the unaffected portions i.e., non-necrotic (NN) stem tissues were cut with a new razor blade and subjected to estimation of protein, total carbohydrate, ascorbic acid, catalase, and guaiacol peroxidase by adopting the methods described above. RC tissues after 20 days of recovery were subjected to the same analyses.

2. Scanning electron microscopy

SEM studies were also conducted on NN, EN and AN tissues of 0.13, 0.25 and 2.54 mg L\(^{-1}\) Cu. In the case of plants exposed to 4.58 and 8.14 mg L\(^{-1}\) Cu exposed plants, only EN and AN tissues were taken. Transverse sections (T/S) of NN, EN and AN stem tissues were collected at different periods of exposure duration for different Cu concentrations depending upon the onset of necrosis in plants. Similar stem T/S from control plants were also made. For SEM studies only the stem tissues were collected but not the root and leaf tissues because leaves were shed and roots completely distorted due to the appearance of necrosis.
3. Metal accumulation in necrotic tissues

Metal accumulation patterns in NN, EN and AN tissues of 0.13, 0.25, 2.54 and 4.58 mg L\(^{-1}\) Cu exposed plants along with RC plants from 2.54 (RC1) and 4.58 mg L\(^{-1}\) (RC2) were also estimated. NN, EN and AN stem tissues were collected at different periods of exposure duration for different Cu concentrations depending upon the onset of necrosis in plants. Samples of control stem were also collected. Stem, root and leaf of RC plants were collected after 20 days of recovery. For metal analysis only the stem tissues were collected. Root and leaf tissues were not analyzed because of poor or no development of root and leaf tissues in plants exposed to Cu. The collected tissues were analyzed for Cu accumulation by atomic absorption spectrometry.

Besides the tissue samples, HS medium at AN and EN stages exposed to 0.25 and 2.54 - 4.58 mg L\(^{-1}\) of Cu were collected analyzed in flame atomic absorption spectrophotometer (Perkin Elmer 3110) for Cu analysis (Gupta, 1996).

II. Nickel

1. Biochemical analysis

Plants were subjected to 4.02 and 7.14 mg L\(^{-1}\) of Ni and the appearance of necrosis was monitored by visual observation. Six replicates were used for studying the appearance of necrosis in Ni exposed plants along with the control plants. Since the appearance of necrosis in Ni exposed plants was very uncertain, only protein and carbohydrates analysis was made. NN, EN and AN tissues of the plants exposed to 7.14 mg L\(^{-1}\) of Ni were taken for protein and total carbohydrates analysis depending upon the appearance of necrosis in the respective Ni concentrations.
2. Scanning electron microscopy

SEM studies were conducted taking AN tissues of 4.02 mg L\(^{-1}\) Ni and EN and AN tissues of 7.14 mg L\(^{-1}\) of Ni exposed plants along with control stem tissues. Transverse sections of EN and AN stem tissues were collected at different points of exposure duration for different Ni concentrations depending upon the onset of necrosis in plants along with control stem samples.

3. Metal accumulation in necrotic tissues

Metal analysis was done with NN, EN and AN tissues of 7.14 mg L\(^{-1}\) Ni exposed plants along with control. Since in case of Ni exposed plants NN tissue was unable to survived, no RC tissues could consequently be obtained. NN, EN and AN tissues of 7.14 mg L\(^{-1}\) Ni along with control stem tissues were subjected to acid digestion and dissolved in distilled water as described above. The resultant solution was used for metal analysis.

Besides this HS medium at AN stage in 7.14 mg L\(^{-1}\) of Ni was analyzed in flame atomic absorption spectrophotometer (Perkin Elmer 3110).

G. STATISTICAL ANALYSIS

Statistical analysis was done using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test at \(p<0.05\). In cases where Tukey test showed lack of significant differences, the slightly less rigorous LSD test at \(p<0.05\) was used to check the significance of difference between the groups using SPSS 20 statistical software for Windows.