MATERIALS AND METHODS

In order to carry out the study on 'Morphological and Physiological responses of *Withania somnifera* to heavy metals (Ni and Cd) and UV-B irradications,' different methods have been used and to follow these methods different materials had to be used. The details of these methods and materials have been mentioned in this chapter.

3.1 Experimental Site:-

The study was performed at Department of Biological Sciences, Sam Higginbottom Institute of Agriculture technology and sciences (SHIATS), Allahabad (Uttar Pradesh), situated in this eastern plains of India at 24° 97' N latitutde and 82° 21' longitude and 96m above mean sea level during 2007 to 2009.

3.2 Climate and Weather Condition:-

The region has moist, sub humid climate dominated by tropical monsoonic character with an annual average rainfall of 959 mm, maximum temperature of 40°C, and minimum temperature of 8°C and mean relative humidity of 63%. The one year time span is divided into three distinct season: summer (April to June), rainy (July to September) and Winter (November to February). March and October constitutes transitional months between winter and summer and between rainy and winter season, respectively.

The metrological data including the weekly average of maximum and minimum temperature, relative humidity and rainfall recorded at Allahabad during the period of experiment in presented data and in Fig 3.2 and in Appendix Table – 3.2.1, 3.2.2.
3.3 Soil Analysis:

Soil samples of about 20g were randomly collected in triplicate from different pots, samples thus collected from different post were mixed separately to get up a composite soil stock. The soil samples were air dried and sieved through a 2mm mesh screen for analyzing various soil parameters. The details of methodology are as follows: 3.3.

3.3 Soil Analysis :

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Results</th>
<th>Method employed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil pH</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td>Available Nitrogen (kg/ha)</td>
<td>197.69</td>
<td>200</td>
</tr>
<tr>
<td>Available Phosphorus (Kg/ha)</td>
<td>22.13</td>
<td>25.00</td>
</tr>
<tr>
<td>Available Potassium (Kg/ha)</td>
<td>119.45</td>
<td>121.00</td>
</tr>
<tr>
<td>Available Sphur (mg/g)</td>
<td>12.70</td>
<td>13.00</td>
</tr>
<tr>
<td>Available Ni (mg/g)</td>
<td>12.4</td>
<td>14.9</td>
</tr>
<tr>
<td>Available Cd (mg/g)</td>
<td>11.2</td>
<td>12.6</td>
</tr>
</tbody>
</table>

3.3.1 Soil moisture content:-

For soil moisture, 5g fresh soil samples were kept in oven at 105°C for 48 hrs and then weighed. Moisture content in soil was calculated by following formula:

\[
\text{Soil Moisture Content (\%) = \frac{\text{Fresh weight-Oven dry weight}}{\text{Fresh weight}} \times 100}
\]
3.3.2 **pH:** Soil pH as measured in the suspension of 1:5 (soil: water, W/V) with the help of a photo volt pH meter using a glass electrode.

3.3.3 **Organic Carbon:** The percent organic carbon was determined by Walkely and Block's rapid titration method described by Piper (1966).

3.3.4 **Total Nirtogen:** For determining the available phosphorus content, 1g soil sample with 200 ml of 0.002 M H₂SO₄ was mixed in a horizontal shaker for 30 minutes. The suspension as filtered through a whatman -44 filter paper and the content was determined by using phosphomolybdcic blue colorimetric method of Jackson (1958).

3.3.5 **Bulk Density and Porosity:** Bulk density was estimated by taking out a core of undistributed soil by metal tube. The soil was oven dried and light was determined. The volume of soil was calculated by measuring the volume of tube ($\pi r^2 h$). The bulk density was calculated by dividing the oven dry weight of soil samples (g) by volume of the soil.

\[
\text{Bulk density} \ (g/cm^3) = \frac{\text{Dry wt of the soil samples}}{\text{Volume of the soil}}
\]

Percent porosity was estimated by using the following formula:

\[
\text{Porosity} \ (%) = 100 - \left( \frac{\text{Bulk density} \times 100}{2.65} \right)
\]

Where 2.65 is the standard particle density.

3.3.6 **Exchangeable Ca^{2+}, Na^+ and K^+ :** For estimating exchangeable bases (Ca^{2+}, Na^+ and K^+) in the soil, repeated leaching was followed (Jackson, 1958). A 10g air dried soil sample, mixed with 50ml 1N ammonium acetate (CH₃COONH₄) solution, was shaken on a horizontal shaker for 30 minutes, stored overnight and then filtered. The process was repeated to leach out the exchangeable bases in the soil sample. The contents of Ca^{2+}, Na^+ and K^+ (%) in the filtrate were determined with a Flame photometer, type 121 (Systronics, India).
3.3.7 Sulphate-Sulphur: For extraction of soil $\text{SO}_4^{2-}$-S, the method of Chesnin and Yien (1950) was followed to a 20g of air dried soil sample, 100 ml of Morgan's extradition solution (100 g of NaOAC and 30 ml of 99.5% HOAC dissolved and mixed in 500 ml of water and volume made to one litre) was added. The suspension was shaken for one and half hours, and then filtered through whatman-42 filter paper. The $\text{SO}_4^{2-}$-S content was determined in the filtrate by using the turbid-ditrimetric method given by Rossum and Villaruz (1961).

3.3.8 Organic matter: For estimating organic matter in the soil, 1g of soil sample mixed with 10 ml 1N $\text{K}_2\text{Cr}_2\text{O}_7$ and 20 ml of $\text{H}_2\text{SO}_4$ (96%) and kept it for 30 min and then, add 200 ml distilled water, 10 ml orthophosphoric acid (85%) and 1 ml diphenylamin indicator. The organic matter content was determined by titrating this solution against ferrous ammonium sulphate 0.5N till colour changed from blue to green.

\[
\text{Organic matter (％) } = \frac{10 \times (B-T) \times 0.003 \times 100 \times 1.3 \times 1.724}{B \text{ wt of soil (g)}}
\]

Where, B-blank and T-sample

3.4 Heavy metal determination:

Heavy metal (Cd and Ni) concentration in the soil were determined by extracting them in 0.0005 M DTPA (diethylene trimine pentoacetic acid) extracting solution (pH 7.3). To a 10 g of air dried soil, 20 ml of DTPA solution was added. The suspension was shaken for 2 hrs and filtered through whatman 42 filter paper into a plastic bottle. The concentrations of heavy metals were determined by using Atomic Absorption Spectrophotometer (Perkin-Elmer, USA).

3.5 Experiment plant:

Aswagandha (Withania somnifera) seeds were obtained from a certified seed store / nursery in Allahabad.
3.6 Raising of plants:

The soil was prepared by mixing farmyard manure in accordance to normal agricultural practices to avoid any change in edaphic conditions. N, P and K were given in form of urea, single phosphate and murate of potash, respectively. Each pot was filled with 5 kg air dried soil. For each treatment there were three replicates.

3.7 Detailed technical programme:

1. Number of Treatments - 15 (14 + Control)
2. Number of Replicates - 03
3. Total number of pots - 45
4. Total number pots in each block - 15
5. Statistical design - RBD
6. Plant - Withania somnifera (Ashwagandha)

3.8 Experimental design:

Uniform seeds were grown in soil pot culture in 45 pots. The experiment was carried out using treatments and one control. Cadmium was applied as CdSO₄ at the rate of 2 mg Kg⁻¹ and 4 mg Kg⁻¹ soil and Ni as NiCl₂ at the rate of 2 mg Kg⁻¹ and 4 mg Kg⁻¹ soil. Combination of Cd and Ni was applied at the rate of 2 mg Kg⁻¹ soil for each of the heavy metals.
3.9 Details of treatments: The details of the treatment are given below:

3.9 Treatment Description:

<table>
<thead>
<tr>
<th>Notation</th>
<th>Treatment level</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀ (Control)</td>
<td>Untreated (normal)</td>
</tr>
<tr>
<td>T₁</td>
<td>2 mg kg⁻¹ Cd</td>
</tr>
<tr>
<td>T₂</td>
<td>4 mg kg⁻¹ Cd</td>
</tr>
<tr>
<td>T₃</td>
<td>2 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₄</td>
<td>4 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₅</td>
<td>2 mg kg⁻¹ Cd + 2 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₆</td>
<td>2 mg kg⁻¹ Cd + 4 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₇</td>
<td>4 mg kg⁻¹ Cd + 2 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₈</td>
<td>4 mg kg⁻¹ Cd + 4 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₉</td>
<td>UV-B (120 min day⁻¹)</td>
</tr>
<tr>
<td>T₁₀</td>
<td>UV-B (120 min day⁻¹) + 2 mg kg⁻¹ Cd</td>
</tr>
<tr>
<td>T₁₁</td>
<td>UV-B (120 min day⁻¹) + 4 mg kg⁻¹ Cd</td>
</tr>
<tr>
<td>T₁₂</td>
<td>UV-B (120 min day⁻¹) + 2 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₁₃</td>
<td>UV-B (120 min day⁻¹) + 4 mg kg⁻¹ Ni</td>
</tr>
<tr>
<td>T₁₄</td>
<td>UV-B (120 min day⁻¹) + 2 mg kg⁻¹ Cd + 2 mg kg⁻¹ Ni</td>
</tr>
</tbody>
</table>

Note:

C = Control

Cd = Cadmium, Source of Cadmium = Cadmium Sulphate (CdSO₄)

Ni = Nickel, Source of Nickel = Nickel Chloride (NiCl₂)

UV-B = Ultraviolet-B rays. (120 min day⁻¹)
3.10 Set up and Standardization of UV-B treatment system under field conditions:

Supplemental UV-B was provided artificially by Q Panel UV-B 313 fluorescent lamps (Q panel, Cleveland, Ohio, USA). Banks of 3 lamps (120 cm long) fitted 30 cm apart on a steel frame were suspended above and perpendicular to the planted rows. The 30 cm distance between the top of the plant canopy and UV-B lamps was kept constant by adjusting steel frame. Plants were irradiated after germination for 2 h day$^{-1}$ in the middle of the photoperiod till the maturity of crops. The lamps were covered by either 0.13 mm cellulose diacetate filter to provide UV-A + UV-B or covered with 0.13 mm polyester filters to the supplemental UV-B. Aluminium reflectance strip was used to avoid scattering of UV rays from upper side of the lamps. Filters were changed frequently to avoid aging effects on the spectral transmission of UV-B.

The UV-B irradiance at the top of the plant canopy under the lamps was measured with an ultraviolet intensity meter (UP Inc. San Gabriel, USA). The readings were converted to UV-B$_{BE}$ values by comparing with the spectro Power Meter (Scientech, Boulder, USA). Plants under polyester filter lamps received only ambient UV-BC 8.6 KJm$^{-2}$ UV-B$_{BE}$) on the summer solstice weighted against generalized plant response action spectrum of Caldwell (1971). The plants beneath cellulose diacetate film received UV-B$_{BE}$ (ambient + 7.1 KJm$^{-2}$) that mimicked 20% reduction in stratiopheric ozone at Allahabad (20° 47' N) during clear sky condition on the summer solstice (Green et al., 1980) normalized at 300 m.
3.11 Observation Recorded:

3.12 Morphological Characteristic:

3.12.1 Shoot Length (cm):

The height of the plants from each pots was measured in cm with the help of measuring tape from ground level to tip of the shoot at 30, 60 and 90 days after sowing. The average height of plants of each replication was recorded and subjected to statistical analysis.

3.12.2 Root Length (cm):

The root of plants were selected and measured whole length of root up to growing tips in natural condition. The observation of the plants were recorded at 90 days after showing. The average Length was then calculated for each observation.

3.12.3 Number of branches per plant:

Number of branches per plant was counted from all plants from each replicate of all the treatment at 30, 60 and 90 days after sowing. The average number of branches per plant of each replication was recorded and subjected to statistical analysis.

3.12.4 Number of leaves per plant:

Observation on the number of leaves per pot was recorded from tagged plants of each pot, the number of leaves was recorded from all plants from each replication of all the treatment at 30, 60, and 90 days after sowing and then the average number of leaves per plant was determined.
3.12.5 Fresh weight (g plant⁻¹):

Five healthy and uniform plants from each treatment were sampled and washed carefully. Precautions were taken against any loss and then surface water in dried with blotting paper. Then the plants were weighed directly on electronic balance.

3.12.6 Dry weight (g plant⁻¹):

Dry weight of plant was recorded on 90 and days after sowing. For this observation plants were uprooted randomly from each pot. The plants were uprooted carefully and kept in sample drier for drying at 70 ± 2 °C temperature. After 48 hours, when plants were completely dried, the weights of those dried plants were recorded.

3.12.7 Fruit Yield : Yield of fruit at 90 days after showing.

3.13 Physiological and Biochemical characteristic :

3.13.1 Photosynthetic pigments :

The chlorophyll and carotenoid contents expressed as mg g⁻¹ fresh leaf were measured by using the methods of Machalachalan and Zalik (1963) and Duxbury and Yentsch (1956), respectively

Reagent : 80% acetone v/v.

Procedure:

For chlorophyll and carotenoid determinations, a 0.1 g leaf sample was placed in 10 ml of 80% acetone in a test tube and kept in overnight in a refrigerator at 4°C. It was then homogenized and centrifuged at 6000 x g for 15 minutes. The optical densities of the supernatant were measured at 480, 510, 645 and 663 nm wavelengths. The contents of chlorophyll a and b and carotenoid were calculated by using the following formulae:
Chl. a \( (\text{mg g}^{-1} \text{ fresh leaf}) \) = \( \frac{2.3 \times D_{663} \sim 0.86 \times D_{645} \sim V}{d \times 1000 \times W} \)

Chl. b \( (\text{mg g}^{-1} \text{ fresh leaf}) \) = \( \frac{9.3 \times D_{645} \sim 0.6 \times D_{663} \sim V}{d \times 1000 \times W} \)

Carotenoid \( (\text{mg g}^{-1} \text{ fresh leaf}) \) = \( \frac{0.6 \times D_{480} \sim 0.49 \times D_{510} \sim V}{d \times 1000 \times W} \)

Where,

\( V = \) volume of extract (ml)

\( d = \) length of light path (cm)

\( w = \) dry weight of leaves taken (g)

Total chlorophyll = ch.la+chl.b

3.13.2 Anthocyanin:

Anthocyanin content in leaves was measured following the method of Beggs and Wellmann (1985).

Reagents:

Propanol, hydrochloric acid and water (18:1:81 v/v) mixture

Procedure:

For the estimation of anthocyanin content, 100 mg leaf sample was homogenized in 100 ml propanol, hydrochloric acid and water mixture (18:1:18, v/v) The solution was boiled for 1.5 min and then kept for overnight. The mixture were centrifuged at 8000x g for 10 min and the optical densities of the extract were measured at 535 and 650 nm on a spectrophotometer. Total amount of anthocyanin was obtained by using the following formula:
Anthocyanin (mg g\(^{-1}\) fresh leaf) = \( \frac{A_{535} - 2.2A_{650}}{W} \times V \)

Where,

\( V \) = ml volume of extract

\( W \) = g fresh weight of leaf.

3.13.3 Flavonoid content :

Flavonoid content was determined by the method described by Flint et al., (1985).

**Reagents:** Ethanol and acetic acid (99:1, v/v)

**Procedure:**

Flavonoids were extracted by homogenizing 0.1 g fresh leaf samples in 100 ml ethanol and acetic acid mixture. Solution was boiled for 2 min and then cooled and centrifuged for 10 to 15 min at 8000 x g. The absorbance of the extract was measured from 250 to 350 nm wavelengths on UV-VIS Spectrophotometer and resulting absorbance profile was plotted as described by Flint et al., (1985). Flavonoid levels were represented and flavonoid absorbance per mass of leaf (A mg\(^{-1}\) fresh wt).

3.14 Enzyme activities :

Superoxide dismutase, catalase and peroxidase enzyme activities were determined using the methods of Giannopolitis and Kies (1977), Kar and Mishra (1976) and Britton and Mehley (1955), respectively.
3.14.1 SOD activity:

Reagents:

1. Extracting Solution

(I) EDTA Phosphate buffer

(i) 0.1 M K$_2$HPO$_4$ = Dissolved 1.74 mg in 100 ml distilled water

(ii) 0.1 M EDTA = Dissolved 3.72 g in 100 ml DW

This is extracting solution.

(I) Riboflavin (1.3 \( \mu \)M) : Dissolved 48.9 mg in 100 ml distilled water 0.1 ml of this solution was diluted to 100 ml with distilled water.

(II) Methionine (13 \( \mu \)M) : Dissolved 1.1939 g in ml distilled water.

(III) Nitroblue tetrazolium (NBT) (63 \( \mu \)M): Dissolved 5.15 mg in 100 ml distilled water. Take 10 ml of this solution and diluted with 90 ml distilled water.

(IV) Na$_2$CO$_3$ (0.05 M) = Dissolved 0.53 g in 100 ml distilled water.

Procedure for extraction:

50 mg of fresh root, stem and leaf samples were homogenized with 2 ml of extracting solution.

For the estimation of superoxide dismutase activity, 3 ml of assay mixture containing 0.5 ml of 1.3 \( \mu \)M riboflavin, 0.5 ml of (13 \( \mu \)M) methionine 0.5 ml of 63 \( \mu \)M NBT, 0.5 ml of 0.05 M Na$_2$CO$_3$, 0.9 ml of distilled water and 0.1 ml of crude enzyme extract was incubated for 10 min at 25°C under illumination from fluorescent lamp. The increase in absorbance due to formazan formation was read at 560 nm. Under these conditions, the increase in absorbance in the absence of
the enzyme was taken as 100% and 50% initiated was taken a equivalent to one unit of SOD activity.

3.14.2 Catalase and Peroxidase activities:

Reagents:

1. Extraction solution: 0.1 M cold phosphate buffer (pH 7) containing 5 mM cystein.
   a. NaOH solution (0.2 M): Dissolved 0.8 g NaOH in 100 ml distilled water.
   b. Na₂HPO₄ solution (0.2 M): Dissolved 3.5598 g Na₂HPO₄ in 100 ml distilled water.
   c. Added 'x' ml of 0.2 M NaOH solution to 50 ml of 0.2 M NaHPO₄ and diluted to 100 ml according to the following table.

<table>
<thead>
<tr>
<th>NaOH (xml)</th>
<th>3.5</th>
<th>5.8</th>
<th>9.1</th>
<th>13</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.8</td>
<td>6.0</td>
<td>6.2</td>
<td>6.4</td>
<td>6.6</td>
<td>6.8</td>
<td>7.0</td>
<td>7.2</td>
<td>7.4</td>
</tr>
</tbody>
</table>

2. Cysteine (5 mM): Dissolved 0.006078 g in 10 ml of phosphate buffer.

3. Phosphate buffer (300 μM): 30 ml of phosphate buffer was diluted up to 100 ml with distilled water.

4. Phosphate buffer (125 μM): 12.5 ml of phosphate buffer was diluted up to 100 ml with distilled water.

5. Pyrogallol (50 μM): Dissolved 0.01261 g pyrogallol in 100 ml distilled water (1 mM). 5 ml of this solution was diluted up to 100 ml with distilled water.
6. **H₂O₂ solution (100 mM):** 1.12 ml H₂O₂ was diluted to 100 ml with distilled water (100 mM). 1 ml of this solution was again diluted up to 100 ml with distilled water (1 mM). 10 ml of 1 mM solution was diluted to 100 ml with distilled water.

7. **H₂O₂ solution (50 μM):** 5 ml of 1 mM H₂O₂ solution was diluted to 100 ml with distilled water.

8. **H₂SO₄:** 2% and 5% (v/v)

9. **KMnO₄:** 0.02 N

10. **Diethyl ether**

Procedure of extraction:

A 100 mg fresh leaf sample was homogenized with 10 ml 0.1 M cold phosphate buffer containing 5 mM cysteine at 4°C. The extract was centrifuged and the supernatant was used for determining catalase and peroxidase activities.

3.14.3 Catalase activity:

For the estimation of catalase activity, 5 ml aliquot of the assay mixture containing 3 ml of 300 μM phosphate buffer (pH7), 1 ml of 100 μM H₂O₂ and 1 ml of diluted enzyme extract was incubated for 1 min at 25°C. Ten ml of 2% H₂SO₄ (v/v) was added to terminate the reaction and the residual H₂O₂ was titrated against 0.02 N KMnO₄ unit a faint ping colour persisted for a few second. A blank was run likewise in which the enzyme activity was terminated at zero minute. The catalase activity was expressed as μM H₂O₂ decomposed min⁻¹g⁻¹ fresh leaf.

\[
\text{Catalase activity} = \frac{(\text{Final reading} - \text{Initial reading}) \times \text{Volume of extracting solution}}{\text{Volume of enzyme extract solution} \times \text{Weight of leaf sample}}
\]

3.14.4 Peroxidase activity:
For peroxidase activity, 5 ml of assay mixture containing 2 ml of 125 μM phosphate buffer (pH7), 1 ml of 50 μM pyrogallol, 1 ml of 50 μm H₂O₂ and 1 ml enzyme extract was incubated for 5 min at 25°C. After 5 min, the reaction was terminated by adding 0.5 ml of 5% H₂SO₄. The purpurogallin formed in the reaction was extracted with 10 ml ether and its quantity was determined spectrophotometrically at 430 nm. A blank was run likewise in which the enzyme activity was terminated at zero minute. The peroxidase activity was expressed as μM purpurogallin formed min⁻¹ g⁻¹ fresh leaf.

\[
\text{Peroxidase activity} = \frac{R \times V_E}{V \times W} \times 2.47
\]

Where,

- \( R \) = Optical density
- \( V_E \) = Volume of enzyme extract
- \( V \) = Volume of extraction solution
- \( W \) = Weight of leaf sample

3.15 Ascorbic acid content:

The method of Keller and Schwager (1977) was used for the extraction and determination of ascorbic acid.
Reagents:

1. **Extraction solution**: Dissolved 5 g oxalic acid and 0.75 g EDTA in one liter double distilled water.

2. **DCPIP (2, 6- dichlorophenol indophenol) solution**:
   i. **Stock Solution**: Dissolved 100 mg of DCPIP in 500 ml of distilled water and heated up to 80°C until it totally dissolved; then cooled and diluted to one liter with distilled water.
   
   ii. **Working solution (20 µg ml⁻¹)**: 20 ml of stock solution was diluted up to 100 ml with distilled water.

3. **Ascorbic acid solution (1%)**: Dissolved 1 g ascorbic acid in 100 ml distilled water.

Procedure:

Extraction: A 500 mg fresh leaf sample was homogenized in a ice bath with 20 ml of extracting solution. The homogenate was centrifuged at 6000xg for 15 min. 1 ml of the supernatant and 5 ml of working 2, - dichlorophenol- indophenol solution was mixed with constant shaking and the O.D. of the ping solution (Es) was determined at 520 nm wavelength. Now one drop of 1% aqueous ascorbic acid solution was added in order to bleach the ping colour of the dye completely and O.D. of the bleached solution (Et) was measured at the same wavelength. For estimation of blank (Eo), 1 ml extracting solution and 5 ml DCPIP solution and 1 drop of ascobric acid solution was mixed together and O.D. was measured at the same wavelength.

A calibration curve was prepared by using 1% aqueous ascorbic acid solution which as diluted to obtain varying concentrations. The total amount of ascorbic acid was calculated by using the following formula:

\[
\text{Ascorbic acid (mg g}^{-1} \text{ fresh leaf)} = \frac{[\{Eo-(Es-Et)} \times V\}] / V \times W \times 1000
\]
Where,

\[ W = \text{weight of leaf taken (g)} \]
\[ V = \text{total volume of the mixture (ml)} \]
\[ v = \text{supernatant taken for analysis (ml)} \]

Value of \( (E_0 - (E_s - E_t)) \) is estimated by the standard curve.

3.16 Phenols :

The method of Bray and Thorpe (1954) was used for the extraction and determination for total phenolics content.

Reagents :

1. 70% acetone (v/v)
2. Sodium carbonate (20% w/v)
3. Folin ciocaleteu reagent (1N)

Procedure :

For phenol determination, 100 mg fresh leaf sample was homogenized with 10 ml of 70% acetone and the suspension was centrifuged at 6000 xg for 10 minutes. To 1 ml of supernatant in a test tube, 1 ml of folin-ciocaleteu reagent and 2 ml of \( \text{Na}_2 \text{CO}_3 \) (20% w/v) solution were added, and the final volume was made up to 10 ml with distilled water. This mixture was then heated in a boiling water bath for one minute and cooled to the room temperature before the O.D. of this blue colored solution was measured at 650 nm wavelength. The \( \mu \text{g} \) phenol content at a given O.D. was determined with a standard curve prepared with known amount of quinone. The mg phenol g\(^{-1}\) fresh leaf was calculated by using the formula given by Bray et al. and described by Bray and Thorpe (1954).
Phenol content = \[ \frac{\mu g \text{ phenol} \times V}{W \times 1000 \times V_1} \]  

(mg g\(^{-1}\) fresh leaf)

Where,

\(\mu g\) phenol is the concentration obtained for the standard curve

\(V\) = total volume of the mixture

\(V_1\) = ml supernatant taken for analysis

\(W\) = weight of leaf sample

3.17 Reducing sugars:

18 ml of supernatant was diluted up to 48 ml with distilled water. In 0.5 ml of aliquot, 1 ml of 5% phenol was added and kept for 10 minutes. Now 5 ml of conc H\(_2\)SO\(_4\) was added, with shaking and allowed to stand for 10 min in a water bath at 25-30\(^0\)C. After this, optical density of the solution was taken at 480 mm.

3.18 Protein:

Protein was estimated by following the method of Lowry et al. (1951).

1. **Reagent A:** 2% Na\(_2\)CO\(_3\) in 0.1N Na OH.

2. **Reagent B:** 0.5% CuSO\(_4\). 5H\(_2\)O in 1% potassium sodium tartarate.

3. **Alkaline copper solution (Reagent C):** 50 ml of reagent A was mixed with 1 ml of reagent B, prior to use.

4. **Folin-ciocalteau reagent (reagent D):** Mixed in the ration 1:1 with distilled water prior to use.
5. **Protein Solution** :

i. **Stock solution**: 50 mg of bovine serum albumin was dissolved in 50 ml distilled water.

ii. **Working solution (200 µg protein/ml)**: 10 ml of stock solution was diluted with 50 ml distilled water.

6. **0.1 M sodium phosphate buffer (pH 7.2 and 7.5)**

<table>
<thead>
<tr>
<th>X(ml)</th>
<th>Y(ml)</th>
<th>pH</th>
<th>Diluted up to 200 ml with distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>72</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>84</td>
<td>7.5</td>
<td></td>
</tr>
</tbody>
</table>

X= 0.2 M solution of monobasic sodium phosphate.

Y= 0.2 M solution of dibasic sodium phosphate.

**Procedure:**

**Extraction of protein**: 500 mg of sample was ground with a pestle and mortar in 5 to 10 ml of buffer. The sample was centrifuged at 5000 × g for 5 min and supernatant was used for protein estimation.

**Estimation of protein**: 0.2, 0.4, 0.6, 0.8, and 1 ml of the working solutions were taken into a series of test tubes. 0.1 ml and 0.2 ml of the sample extract was taken in another two test tubes. The volume was made up to 1 ml in all the test tubes with distilled water. One ml of distilled water was taken as blank in the tube. 5 ml of reagent C was added in each test tube and after mixing allowed to stand for 10 minute. Afterward, 0.5 ml of reagent D was added in each test tube. The test tubes were incubated after mixing at room temperature in dark for 30 minutes, until blue colour developed. Absorbance was measured at 650 nm wavelength. A standard curve was prepared with known concentrations of
albumin using above mentioned method. The amount of protein was expressed in mg g\(^{-1}\) fresh leaf.

3.19 Statical Analysis and Presentation of Data:

The data recording during the course of the study were subjected to Statical Analysis as per method of ‘Analysis of Variance’ (ANOVA). ANOVA test was conducted to test the significant effects of UV-B treatment, heavy metals (Cd and Ni) treatment, plant age and their interaction data recorded. The quantitative changes observed for various parameters were tested on the basis of ‘F’ test and C.D. at 5% level between means of significance by Duncan’s Multiple Range Test (DMRT). For the testing the hypothesis, ANOVA table was used.

3.19 Table: Skeleton of ANOVA Table.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.S.</th>
<th>F(cal)</th>
<th>F(tab) at 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Due to replication</td>
<td>r-1</td>
<td>R.S.S.</td>
<td>R.S.S./r-1</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Due to treatment</td>
<td>t-1</td>
<td>T.S.S.</td>
<td>T.S.S./t-1</td>
<td>MT.S.S./MESS</td>
<td>F(tab), (r-1)(t-1)</td>
</tr>
<tr>
<td>Due to error</td>
<td>(r-1)(t-1)</td>
<td>E.S.S.</td>
<td>E.S.S./ (r-1)(t-1)</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Total</td>
<td>rt-1</td>
<td>T.S.S.</td>
<td>T.S.S./ rt-1</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>
\[ \text{SE} = \sqrt{2 \times \text{emss}} \]

\[ \text{r} \]

C. D. = SE(m)xt (5%) on error d.f. at 5% levels of significant

Where,

\[ r = \text{replication} \]

\[ t = \text{treatment} \]

\[ \text{ss} = \text{sum of square} \]

\[ \text{Rss} = \text{Replication sum of square} \]

\[ \text{Tss} = \text{Treatment sum of square} \]

\[ \text{Ess} = \text{Error sum of square} \]

\[ \text{Tss} = \text{Total sum of square} \]

\[ \text{Mess} = \text{Error mean sum of square} \]

\[ \text{MTi: S.S.} = \text{Mean treatment sum of square} \]
Fig. 3.2.1: Meteorological observations on weekly intervals during the period of investigation (2007-2008).
Fig. 3.2.2: Meteorological observations on weekly intervals during the period of investigation (2008-2009).