The Plant Material

The present study was carried on germplasm of *Eclipta alba* (L.) Hassk., collected from Punjab and adjoining states and raised at the Botanic Gardens, Punjabi University, Patiala. The germplasm thus maintained was characterized for morphological traits, chromosome studies, meiotic behaviour, reproductive biology and RAPD profiling at molecular level. Micro propagation and cultivation trials were also performed. The details of each approach are present below:

Survey and Collection

For collection of plant materials, extensive surveys have been conducted during 2006 covering different places in Punjab, Haryana, Chandigarh, Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh, and Northern Rajasthan as shown in Map (Fig. IV). The experimental material comprised of thirty four accessions of *Eclipta alba* plants which have been raised at the Botanic Gardens, Punjabi University, Patiala. The details of the accessions with data on locality, latitude, longitude and altitude are presented in Table 3.1. The voucher specimens were deposited in the herbarium, Department of Botany, Punjabi University, Patiala, India (PUN).

THE METHODS

3.1 MORPHOLOGICAL STUDIES

All the accessions were evaluated for a number of morphological parameters to make out the different morphotypes. Five randomly selected mature plants from each accession were chosen for recording observations and mean data were used for statistical analysis.

Plant Habit

The plants which grow upright are marked as erect, low growing plants with stem creeping on ground are marked as prostrate and plants with main stem growing upward and lower branches creeping on ground are marked as semi erect plants.
Material and Methods

Plant Height

Plant height was measured as length of the main stem of plant from the surface of soil to the tip of the highest leaf or flowering axis in erect and semierect plants and total spread of the plant in case of prostrate plants.

Stem Colour

It is taken as colour of the main stem and branches.

Number of Nodes per plant

Numbers of nodes from base of the stem to the tip were counted.

Number of Branches per plant

The number of primary branches were counted.

Leaf Colour

The colour of mature leaves was considered and plants were grouped as green and dark green.

Leaf Size

Leaf size was recorded from fully expanded mature leaves in middle of plants. Leaf length was measured from base of attachment of the leaf of mature plant to the tip of the leaf and leaf breadth of the lamina was taken at the widest part of the leaf.

Leaf margin

It is taken as margin of mature leaves.

Inflorescence Size

The size of inflorescence was recorded as the diameter of the mature fully opened flowering head.

Seed Colour

It is the colour of mature seed.
Fig IV: Map showing different areas of collection from North India
### Table 3.1: Data on locality, latitude, longitude and altitude of various accessions of *Eclipta alba* collected from different locations of North India

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>State, District and Locality</th>
<th>Latitude, Longitude/Altitude (m)</th>
<th>Fig. No.</th>
<th>PUN No.</th>
<th>Accession No.</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Punjab, Patiala, Punjabi Univ. Campus</td>
<td>30.36° N, 76.45° E/244</td>
<td>Fig.1</td>
<td>54781</td>
<td>EPBP1</td>
</tr>
<tr>
<td>2.</td>
<td>Punjab, Gurdaspur, Batala</td>
<td>31.82° N, 75.20° E/252</td>
<td>Fig.2</td>
<td>54784</td>
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<tr>
<td>3.</td>
<td>Punjab, Gurdaspur, Bhagowal</td>
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<td>Fig.3</td>
<td>54786</td>
<td>EPBG2</td>
</tr>
<tr>
<td>4.</td>
<td>Punjab, Ludhiana, Sahnewal</td>
<td>30.84° N, 75.95° E/238</td>
<td>Fig.4</td>
<td>54798</td>
<td>EPBL1</td>
</tr>
<tr>
<td>5.</td>
<td>Punjab, Jalandhar, Jalandhar</td>
<td>31.19° N, 75.34° E/228</td>
<td>Fig.5</td>
<td>54799</td>
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<td>6.</td>
<td>Punjab, Ferozpur, Abohar City</td>
<td>30.13° N, 74.20° E/180</td>
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<td>7.</td>
<td>J &amp; K, Jammu, Jammu Univ. Campus</td>
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<td>Fig.7</td>
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<td>9.</td>
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<td>10.</td>
<td>Punjab, Gurdaspur, Dera Baba Nanak</td>
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<td>SPBG2</td>
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<td>11.</td>
<td>Punjab, Gurdaspur, Pathankot</td>
<td>32.17° N, 75.42° E/331</td>
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<td>12.</td>
<td>Punjab, Amritsar, Ramdas</td>
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<td>13.</td>
<td>Punjab, Tarntaran, Tarntarn</td>
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<td>14.</td>
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<td>16.</td>
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<td>Fig.16</td>
<td>54814</td>
<td>SUPM1</td>
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<td>17.</td>
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<td>Fig.17</td>
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<td>19.</td>
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<td>20.</td>
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<td>27.</td>
<td>Punjab, Muktsar, Muktsar</td>
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<td>28.</td>
<td>Rajasthan, Sri Ganga Nagar,</td>
<td>29.91° N, 73.83° E/163</td>
<td>Fig.28</td>
<td>54805</td>
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<td>29.</td>
<td>J &amp; K, Jammu, Jammu Univ. Campus</td>
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<td>Fig.29</td>
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<td>Fig.34</td>
<td>54813</td>
<td>PUPM1</td>
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</table>
3.2 REPRODUCTIVE BIOLOGY

Different aspects of reproductive biology of the three morphotypes i.e. erect, semi erect and prostrate of Patiala populations of *Eclipta alba* has been studied. The observations were taken during the peak blooming period. It includes the following aspects:

3.2.1 Floral Morphology

Detailed studies of various parts of flower head were made using a dissecting microscope. Following characters have been observed regarding the morphology of flowers.

(i) Corolla shapes of Florets

Different corolla shapes of the ray and disc florets have been observed and photographed.

(ii) Number of Ray and Disc Florets

The total number of ray and disc florets per capitulum in three morphotypes of Patiala populations of *E. alba* has been counted by using needle and pair of forceps. For this ten flower heads were taken for each type and mean was calculated.

(iii) Number of seeds per capitulum

The total number of seeds produced per capitulum has been counted in three morphotypes of Patiala populations of *E. alba* by using pair of forceps. For this ten flower heads were taken for each type and mean was calculated.

3.2.2 Flowering phenology

Initiation and duration of phonological events from sprouting of flower buds, anthesis and up to their senescence were studied. Flower phenology was determined by tagging five individuals and recording the observations. The selected plants were kept under careful observation much before the initiation of blooming. For determining the duration of flower development five primordia were marked on each plant and the time taken by each primordium up to the opening of flower was recorded.
3.2.3 Anthesis

Anthesis is the time of opening of flower. It was worked during the peak blooming period, i.e. at two hour interval, from 00:00 hrs to 24:00 hrs in the month of July. For the study of anthesis, the flowers in these plants were carefully observed with the help of a hand lens throughout the course of anthesis. Data on temperature of that time was recorded.

3.2.4 Stigma Receptivity

Stigma receptivity of *E. alba* in the field tested for stigmatic peroxidase activity (SPA) using the method of Kearns an Inouye (1993). Intact styles were placed on glass slide in a drop of 3% hydrogen peroxide and covered with cover slip. Bubble production from the stigma within 2–3 minutes indicate the activity of stigmatic peroxidases and hence receptivity of stigma. Styles tested for peroxidase activity were preserved in 70% ethyl alcohol. At a later date, the styles were digested in 1mol/L NaOH for 1 hour and subsequently stained with aniline blue (Martin, 1959) and mounted on glass slides and observed under fluorescence microscope.

3.2.5 Pollen Characteristics

(a) Pollen Fertility

For determining pollen fertility, glycerooacetocarmine method given by Marks (1954) was used. Mature anthers were collected from freshly opened flowers and squashed in few drops of mixture of glycerin and acetocarmine (1:1) and kept at 60 °C for five minutes. Observations were made after 2 hours. Uniformly stained and well filled pollens were considered to be fertile whereas shriveled, transparent pollen that acquired no stain were counted as sterile. Percentage of pollen fertility was calculated as:

\[
\text{% age pollen fertility} = \frac{\text{Number of fertile pollen}}{\text{Total pollen observed}} \times 100
\]
(b) Compatibility behavior

To study the compatibility behavior, bagging experiment was carried out. To understand the pollination mechanism bagging experiments were carried out in accession PPBP1. Bags of fine cotton cloth were used for bagging which were done to note the mode of pollination of the flowers. In bagging, the buds that were about to open were covered and were left as such till fruit formation.

(c) Pollination system

To study the mode of pollination in *Eclipta* all the insect visitors were carefully observed even the flower heads were observed for small insects inside them. The insect found in the florets were caught, fixed and identified using the expertise of Department of Zoology, Punjabi University, Patiala.

3.2.6 Seed germination

To study the seed germination, about 100 seeds of the three accessions were sown in separate petri dish lined with moist cotton in laboratory conditions in the months of January and July. The germinated seedlings emerged from seeds were counted with the help of pair of forceps.

3.3 CYTOLOGICAL STUDIES

The *Eclipta alba* plants were investigated cytologically by meiotic and mitotic analysis.

3.3.1 Meiotic studies

(a) Fixation of materials

For meiotic studies, young floral buds were fixed in Carnoy’s fixative (6 parts ethanol: 3 parts chloroform: 1 part glacial acetic acid) for 24 hours and then stored in rectified spirit until use.
(b) Preparation of slides

For chromosomal preparations, anthers were smeared in 1% acetocarmine (prepared by dissolving BDH carmine in 45% acetic acid). Desirable slides were made permanent following McClintok’s (1929) method of dehydration after gradation through 1:1, 1:3, 1:9 grades of acetic acid and ethanol and finally 100% ethanol. Slides are mounted in eupaerol. Chromosome number was confirmed from observations made from several pollen mother cells (PMCs) at different stages.

(c) Observations and photography

The different phases of meiosis were observed under the microscope. Photomicrographs were taken using Lieca digital photomicrography system and Nikon 80i Eclips microscope.

3.3.2 Chiasma Frequency Studies

Chiasma frequency was recorded from PMCs at diakinesis and metaphase-I stages throughout the year with an interval of two months on all the three morphologically distinct accessions of Patiala population i.e. erect, semierect and prostrate growing at Botanic gardens, Punjabi University, Patiala. To study the seasonal variation in the chiasma frequency the buds were fixed from randomly selected five plants of each type during first week of January, March, May, July, September and November during 2008. Pollen mother cells were analyzed from each of these accessions. Chiasma frequency was estimated from ten pollen mother cells (PMC) scored in each plant and is expressed as chiasmata per cell and per bivalent. Two measures of recombination i.e. recombination index and number of excess chiasmata, were calculated from chiasma frequency and number of chromosomes following Koella (1993). The recombination index RI= n(1+x) where n is haploid chromosome number and x is number of chiasmata per bivalent and excess chiasmata is calculated by subtracting the number of bivalents from the number of chiasma in a cell.
3.4 MOLECULAR STUDIES

Different accessions of *E. alba* were analysed for genetic diversity based on randomly amplified polymorphic DNA (RAPD) analysis. The details are as follow:

3.4.1 Plant Material for DNA preparation

Genomic DNA of different accessions of *E. alba* were isolated using CTAB (Cetyltrimethyl ammonium bromide) method as proposed by Doyle and Doyle (1987).

Reagents, Chemicals and Solutions

1. **Liquid Nitrogen**
2. **Cetyl trimethyl ammonium bromide (CTAB) 20% solution**: 2 grams of CTAB was dissolved in sterile water and volume was made upto 100ml and autoclaved.
3. **Ethylene diamine tetra acetic acid (EDTA) 0.5M solution (pH-8.0)**: 18.62 grams EDTA was dissolved in sterile distilled water and pH was adjusted to 8.0 with 1M NaOH. The volume was made 100 ml and the solution was autoclaved.
4. **Tris HCl Buffer (pH-8.0)- 1M Solution**: 12.11 grams of Tris salt was dissolved in sterile distilled water and pH adjusted to 8.0 using 1N HCl. The volume was made upto 100 ml and then autoclaved.
5. **Sodium chloride (NaCl) 5M solution**: 292.2 grams of NaCl was dissolved in sterile distilled water and volume made upto 100 ml and this solution was autoclaved.
6. **2-mercaptoethanol 0.2% Solution**
7. **DNA Extraction Buffer**: The extraction buffer consists of 20% CTAB, 1M Tris HCl (pH-8.0), 5M NaCl, 0.5M EDTA (pH-8.0), 2-mercaptoethanol (0.2%).
8. **Chloroform: Isoamylalcohol (24:1, v/v) mixture**: Chloroform and Isoamylalcohol were mixed in the ratio of 24:1 and stored in an ambered coloured bottle.
9. **Ethanol (70%)**: 70 ml of absolute alcohol was mixed with 30 ml of sterile water to make 100 ml 70% ethanol.
10. **Tris EDTA Buffer (TE):** 10 ml of Tris (1M) buffer and 0.2 ml of EDTA (5M) was mixed with sterile water and volume made up to 100 ml and autoclaved prior to use.

### 3.4.2 DNA Extraction

The DNA isolation was carried out by the following method:

1. Leaves of *E. alba* were washed with sterile distilled water followed by washing with 70% ethanol.
2. Two grams of leaf sample was ground in liquid nitrogen to fine powder in a pre-chilled mortar and pestle. To this 1mg Polyvinylpyrrolidine (PVP) was added.
3. This fine powder was immediately transferred to a centrifuge tube containing preheated 15 ml extraction buffer (CTAB) and 24 µl β-mercaptoethanol to make slurry.
4. The extraction buffer and powder were mixed well and incubated at 60 °C for 1 hour on a water bath with occasional shaking by inverting the tubes.
5. After incubation the mixture was cooled to room temperature and equal volumes of Chloroform: IAA (24:1) was added and mixed gently for at least 15-30 min. Mixture was centrifuged at 10,000 rpm for 10 min.
6. The aqueous phase (supernatant) was transferred to fresh and sterile micro centrifuge tube and DNA was precipitated by adding equal volumes of ice cold isopropanol. Before this 1 ml of 5M NaCl solution was added and mixed well.
7. The tube was stored at -20 °C for overnight for DNA precipitation.
8. The precipitated DNA was collected out by centrifugation at 12,000 rpm for 5 minutes.
9. The supernatant was poured off and the pellet was air dried and washed twice with 70% ethanol and again air dried.
10. The dried DNA pellet was dissolved in TE buffer.
3.4.3 Purification of genomic DNA

Reagents

1. **Phenol : Chloroform : Iso-amyl alcohol mixture (25:24:1):** The mixture was freshly prepared at the time of use. The phenol, chloroform and iso-amylalcohol was mixed in the ratio of 25:24:1 and stored in a bottle.

2. **RNase A Solution (10 mg/ml)**

3. **Sodium acetate (3M Solution):** 30.75 grams of sodium acetate was dissolved in sterile water and volume made up to 50 ml and solution was autoclaved.

The major contaminants in the DNA preparations are RNA, protein and polysaccharides. For various molecular studies purification of DNA is utmost important. In DNA extraction buffer, CTAB helps in elimination of polysaccharides from DNA preparations to a large extent. By treating the samples with RNase, RNA is removed. Extraction with phenol: chloroform following RNase treatment is also used for eliminating RNA and most of the proteins. The protocol for DNA purification is given below:

1. The DNA sample was treated with 5 µl RNase (10 mg/ml stock) and incubated in water bath for 30 min at 37 ºC.

2. Equal volume of mixture of phenol: chloroform: isoamylalcohol (25:24:1) was added. It was thereafter centrifuged at 8000 rpm for 10 min at 25 ºC.

3. The aqueous phase was taken and equal volume of the mixture of chloroform: isoamylalcohol was added and mixed.

4. The aqueous phase was collected and mixed to 1/10 volume of 3M sodium acetate and then mixed double volume of chilled 100% ethanol for precipitation of DNA.

5. DNA was spooled out, washed with 70% ethanol twice and dried at room temperature in a laminar flow for 30 min.

6. Finally, DNA pellet was dissolved in 50 µl of 1X TE buffer and stored at -20 ºC for future work.
3.4.4 DNA Quantification

Reliable measurements of DNA concentration are important for many applications in molecular biology including amplification of target DNA by polymerase chain reaction. DNA quantification is generally carried out by agarose gel analysis.

Reagents
I. Tris-Borate-EDTA (TBE) Buffer – 10X Solution
   Tris base - 108 g
   Boric acid - 55 g
   EDTA - 40 ml

Sterile water was added to the above compounds to make volume 1000 ml.

II. Ethidium bromide (10mg/ml): 10 mg of ethidium bromide was dissolved in sterile water and volume made up to 1 ml. The solution was stored in an amber coloured bottle at 4 °C.

III. Sample Loading Dye (6X)
   Bromophenol Blue (1%) - 0.2%
   Xylene Cyanol (1%) - 0.2%
   Sucrose - 60%
   EDTA (0.5M) - 60 mM

3.4.5 Agarose Gel Analysis
1. Agarose gel (0.8%) was made in 1X TBE buffer and 2 µl ethidium bromide was added. This was poured in gel tray and combs were placed to form wells and allowed to solidify.
2. After solidification of gel, it was transferred to the electrophoresis tank having 1X TBE buffer.
3. 2 µl sample loading dye and 2-5µl of DNA was mixed well and loaded into wells.
Material and Methods

4. Electrophoresis was run at 90V for 30 minutes.

5. The quality of DNA was checked under UV transilluminator with the presence of single compact band.

3.4.6 PCR Amplification

DNA sample was diluted with appropriate amount of sterilized distilled water to yield a working concentration of 100 µg/ml of DNA and stored at 4 °C. Polymerase chain reaction technique was used for amplification of a specific DNA segment using specific primer sequence. The basic PCR requires DNA templates that contains the DNA region to be amplified, two primers which are complementary to DNA regions at the 5’ or 3’ ends, Taq polymerase, dNTPs, buffer solution, magnesium ions. PCR involves three basic steps which constitute a single cycle: (i) Denaturation of target DNA at 92-94 °C for 1 minute (ii) Annealing of the primers to the single-stranded DNA template at 35-38 °C and (iii) Extension of primers by addition of nucleotide to the 3’ end of the primers at 72 °C by the enzyme DNA polymerase.

3.4.7 Protocol of RAPD-PCR for agarose gel electrophoresis analysis

Randomly Amplified polymorphic DNA (RAPD) utilizes a single random decamer primer to amplify genomic DNA. The amplified fragment profile of different genotypes can be used as genetic markers for genetic variability, variety identification and genome mapping. RAPD-PCR reactions were performed according to the protocol by Williams et al. (1990), using the DNA samples isolated from leaves of *E. alba* with RAPD primers using (Bio-rad) Thermal Cycler.

3.4.8 Primer Screening

A preliminary experiment on selected *E. alba* accessions was carried out on 25 primers to select most suitable primers for identification. Eight primers were screened for repeatability, scorability and their ability to distinguish within accessions as shown in Table 3.2 marked with asterisk. Random primers procured from Genei Bangalore Pvt. Ltd India exhibited maximum efficiency of discrimination in terms of resolving power (Prevost and Wilkinson, 1999).
Table 3.2: Primers used for RAPD-PCR of *E. alba*

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<thead>
<tr>
<th>Primer Name</th>
<th>Accession Number</th>
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<td>RPI 6*</td>
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<td>AM773775</td>
</tr>
<tr>
<td>RPI 16*</td>
<td>AM773776</td>
</tr>
<tr>
<td>RPI 17</td>
<td>AM911710</td>
</tr>
<tr>
<td>RPI 18</td>
<td>AM765830</td>
</tr>
<tr>
<td>RPI 19</td>
<td>AM773777</td>
</tr>
<tr>
<td>RPI 20</td>
<td>AM773317</td>
</tr>
<tr>
<td>RPI 21</td>
<td>AM765820</td>
</tr>
<tr>
<td>RPI 22</td>
<td>AM911711</td>
</tr>
<tr>
<td>RPI 23</td>
<td>AM911712</td>
</tr>
<tr>
<td>RPI 24</td>
<td>AM765821</td>
</tr>
<tr>
<td>RPI 25*</td>
<td>AM770054</td>
</tr>
</tbody>
</table>

3.4.9 RAPD-PCR amplification reaction mixture

DNA extracted from each accession was used in subsequent PCR amplifications, which were performed in a programmable thermocycler. A total of 25 µl reaction cocktail was prepared for PCR amplification. Reaction mixture consists of following components. In a sterile 0.5 ml PCR tube, following components were mixed in the order as given below (Table 3.3):
Table 3.3: Reaction mixture for RAPD-PCR of *E. alba*

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTPs (10 mM each)</td>
<td>2.5</td>
</tr>
<tr>
<td>RAPD Primer (15 p moles)</td>
<td>2.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (3U)</td>
<td>1.5</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2.0</td>
</tr>
<tr>
<td>Sterile Distilled water</td>
<td>13</td>
</tr>
</tbody>
</table>

3.4.10. PCR Thermal cycling conditions

The amplification was performed in a Bio-rad thermal cycler. The cycling conditions were as follows:

- **Step (I)** 1 - Cycle
  - Denaturation at 94 °C for 5.0 minutes.

- **Step (II)** 8 - Cycles
  - Denaturation at 94 °C for 45 seconds.
  - Annealing of primers at 35 °C for 1 minute.
  - Chain extension at 72 °C for 1.5 minutes.

- **35 - Cycles**
  - Denaturation at 94 °C for 45 seconds.
  - Annealing of primers at 38 °C for 1 minute.
  - Chain extension at 72 °C for 1 minute.

- **Step (III)** 1 – Cycle
  - Final extension at 72 °C for 10 minutes.
3.4.11 Agarose Gel Electrophoresis

1. Agarose gel of 2.0% was prepared in 1X TBE buffer with visualizing dye ethidium bromide solution (0.5µg/ml).
2. Added 3 µl of gel loading dye to each tube containing the amplified DNA.
3. Samples (10 µl each) were loaded and electrophoresis was carried out at 150V for 1-1.30 hours.
4. Gel was viewed under UV transillumination and photographed.
5. Band position was compared with the amplified DNA ladder.
6. The size of each band was estimated using the DNA molecular weight markers (100bp and 100-5000bp DNA Ladders).
7. Bands were scored and data file was created.

3.4.12 Statistical Data analysis

PCR-RAPD amplified fragments were scored as present (1) or absent (0). The data matrix was then used for molecular analysis NTSYS-PC version (Rohlf, 1993). The data were analyzed by SIMQUAL programme of NTSYS-PC version. The variable binary similarity matrix was prepared using Jaccard’s similarity coefficient by NTSYS (Numerical Taxonomy System of multivariate Programme) computer programme version 2.02e. Dendrogram was prepared by UPGMA (Unweighed pair group method with arithmetical average) analysis.

3.5 BIOCHEMICAL STUDIES

The accessions maintained at Botanical gardens in Punjabi University were subjected to various biochemical studies. Different E. alba plants has been evaluated quantitatively for various phytoconstituents i.e. carbohydrates, proteins, phenols, saponins and wedelolactone content using standard methodology. Analysis of these phytochemicals was performed from the leaves. Various estimations have been made in the months of July and August.
3.5.1 Carbohydrate Content Estimation

Carbohydrate content was estimated in all the accessions by using the method proposed by Ashwell, 1957.

**Reagents:**

Anthrone reagent :  2.0 g/L in concentrated H$_2$SO$_4$

Glucose :  0.1g/L in distilled water

**Preparation of plant extract**

Plant (leaf) extract was prepared by homogenizing 5 g of material in 5 ml of distilled water followed by centrifugation at 5000 rpm. The supernatant was collected and the residue was again suspended by adding 5 ml distilled water and then centrifuged to complete extraction. The supernatant were pooled and volume was adjusted to 10ml.

**Estimation**

To 1 ml of the test solution (leaf extract), 4 ml of anthrone reagent was added and thoroughly mixed. The tubes were then covered with a marble and kept in boiling water bath for 10 minutes. The tubes were allowed to cool to room temperature and the absorbance was read at 620 nm against the blank of distilled water replacing the extract. Standard curve was prepared by using glucose (10-100µg/ml).

**Calculations**

Carbohydrate Content (µg/ml) (a) =

\[
 a = \frac{\text{Absorbance of extract}}{\text{Absorbance of glucose solution}} \times \text{Concentration of glucose solution}
\]

Carbohydrate content of Plant Part (mg/g) =

\[
 a \times \frac{\text{Total volume of the extract}}{1000} \times \frac{\text{Weight of sample taken}}{\text{Weight of sample taken}}
\]
3.5.2 Protein Content Estimation

Protein content of the leaves was estimated using the method given by Lowery et al. (1951).

**Reagents**

- **0.1N NaOH**: 0.4 g NaOH in 100 ml distilled water
- **15% TCA**: 15 g Trichloroacetic acid in 100 ml water
- **Soln. A**: 2.0% Na₂CO₃ in 0.1N NaOH
- **Soln. B**: 0.5% CuSO₄·5H₂O in 1% Sodium potassium tartrate
- **Soln. C**: Prepared by mixing solution A and solution B in the ratio of 50:1 at the time of use
- **Soln. D**: Mixed one part of Folin-Ciocalteau’s phenol reagent and one Part distilled water at the time of use
- **BSA Soln.**: 0.1 g Bovine serum albumin in 1 liter of distilled water

**Preparation of plant extract**

Five grams of fresh plant material was homogenized in 5 ml of 0.1 N NaOH, centrifuged at 3000 rpm and supernatant was collected. The residue was re-suspended in centrifuge again. The two supernatants were pooled and the final volume was adjusted to 10 ml. From this solution, 2 ml of the supernatant was treated with 1 ml of 15% TCA and kept at 4 °C for 24 hrs. Precipitates of protein were formed which were separated by centrifugation at 5000 rpm for 20 min. Supernatant was discarded and precipitate were dissolved in 5ml of 0.1N NaOH and used for estimation.

**Estimation**

5 ml of soln. C was added to 1 ml of protein extract taken in a test tube and mixed thoroughly. The solution was left at room temperature for 10 min. and then 0.5 ml of soln. D was added to it and mixed. After 30 min. absorbance was taken at 520 nm against the blank of distilled water replacing the extract. Protein estimation was made by using standard curve prepared by using BSA (10-100 μg/ml).
Calculations:

Protein content (µg/ml) (a) =

$$a = \frac{\text{Absorbance of extract}}{\text{Absorbance of BSA}} \times \text{Concentration of BSA}$$

Protein content of Sample (mg/g) = \(\frac{a}{1000} \times 5\)

3.5.3 Phenol Content Estimation

Total phenol content estimation has been done on all the accessions of *Eclipta alba* following the procedure of Singleton and Rossi (1965) in the month of October.

Reagents

80% Ethanol

Folin-Ciocalteau reagent

20% Sodium Carbonate

Preparation of Plant Extract

Two gram fresh leaves were homogenised in 80% aqueous ethanol at room temperature and centrifuged in cold at 10,000 rpm for 15 min and the supernatant was saved. The residue was re-extracted twice with 80% ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature.

Estimation

Residue was dissolved in 5 ml distilled water. 100 µl of this extract was diluted with 3ml distilled water and 0.5 ml Folin Ciocalteau reagent was added. After 3 min, 2 ml 20% Sodium carbonate was added and the contents were mixed thoroughly. After 60 min the absorbance of the solution was taken at 650 nm. The results were expressed as mg of gallic acid equivalents per gram weight.
Calculations:

Total Phenol Content (mg/g) =

\[
\frac{\text{Volume of extract} \times \text{Dilution factor} \times \text{Absorbance of extract}}{\text{Absorbance of gallic acid} \times \text{Conc. of gallic acid} \times \text{Weight of samples}}
\]

3.5.4 Saponins Content Estimation

Saponins content was estimated by using the method of Obadoni and Ochuko, 2001.

Reagents

- 20% Ethanol
- Diethylether
- n-butanol
- NaCl

Estimation

The leaf sample of *E. alba* was finely ground and 10 g of each were put into a conical flask to this 200 ml of 20% aqueous ethanol were added. The suspension was heated over a hot water bath for four hour with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 °C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigrously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight. The saponins content was calculated as percentage.

Calculations:

Percentage of Total Saponins =

\[
\frac{\text{Weight of residue}}{\text{Weight of sample taken}} \times 100
\]
3.5.5 Phytate Content Determination

Phytate content in three morphotypes of Patiala populations of *Eclipta alba* was estimated by using the method of Abulude (2005).

**Sample Collection:**

Three morphotypes of *E. alba* were collected and washed under running tap water and with distilled water to remove dirt. The leaves were separated from plants, washed several times in distilled water and allowed to drain and then oven dried at 100 °C for 24 hr. They were then ground in a blender and stored in air tight container prior to analyses.

**Phytate Determination**

Four grams of finely ground leaf sample was soaked in 100 ml of 2% HCl for 3h and then filtered through two layers of hardened filter paper. 25 ml of the filtrate was placed in a 400 ml beaker and 5 ml of 0.3% NH4SCN was added as an indicator. 53.5 ml of distilled water was then added to reach the proper acidity. This mixture was titrated with FeCl3 solution. Equivalent to this, the amount of Phytate P was found by multiplying with a factor of 1.95. This product again multiplied by a factor of 3.55 to convert the result into phytate.

3.5.6. Wedelolactone Content Estimation

Wedelolactone is the active principle compound present in *E. alba* due to which the plant has high medicinal value. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) was carried out to know the concentration of wedelolactone in *Eclipta* by using the method of Yuan et al. (2007). The methanol extract of the shade-dried leaves was analysed by HPLC at a wavelength of 351 nm to estimate its concentration following the standard protocol. The chromatographs were obtained by gradient elution of mobile phases at appropriate wavelength.
Material and Methods

Material

The following materials are needed to carry out the analysis:

- **Standard of Wedelolactone**
- **Apparatus**: HPLC instrument
  - HPLC column – Kromasil C-18, 5µm
  - HPLC injection syringes, 0.45 µm Millipore filter
- **Reagents**: HPLC grade methanol and water

All the Chemicals including HPLC solvents were of analytical grade purchased from Sigma Aldrich. The standard wedelolactone was purchased from Sigma Aldrich (New Delhi, India).

HPLC Conditions

The following HPLC conditions were used

- **HPLC Column**: C-18 Column with dimensions 250 mm x 4.6 mm, 5µm
- **Column temperature**: 35 °C
- **Mobile phase**: methanol- 0.5% acetic acid (55: 45, v/v)
- **Flow Rate**: 1.0 ml/min
- **Injection volume**: 20 µl
- **Detection wavelength**: 351 nm
- **Running time**: 25 min

Preparation of Sample

Leaves of the plants were collected and dried in shade and grounded. One gram of finely ground leaf powder was extracted thrice with 100 ml of methanol for three days. The combined extracts are concentrated to dryness under vacuum at 45 °C. Dried methanol extracts of *E. alba* were redissolved in 5 ml of HPLC grade methanol, filtered through 0.45 µm filter and sonicated for five minutes.
Preparation of Standard solution

Stock solution of wedelolactone 1 mg/ml was prepared in methanol. The standard wedelolactone (Sigma, USA) was dissolved in 1 ml HPLC grade methanol and different amounts were used for preparing four point calibration curve.

Procedure

- Standard stock solutions and samples were prepared.
- A single injection of standard using 0.45 µm filter was made.
- 20 µl of samples were injected with an injector valve.
- A peak for standard was obtained.
- The chromatograph was operated in the gradient mode, using appropriate mobile phase.
- Peak area and retention time for samples and standard was noted.
- Area of the peak corresponding to the retention time of standard was noted.

Calculations

Amount of Wedelolactone was calculated as follows:

\[
\text{Wedelolactone (mg/g)} = \frac{\text{Peak area of sample}}{\text{Peak area of Standard}} \times \text{Concentration of the standard}
\]

3.6 PLANT BIOACTIVITY ASSAY

Antibacterial Activity Assay

Antibacterial activity assay was studied on the three morphotypes i.e. erect, semierect and prostrate of Patiala populations of Eclipta alba.

3.6.1 Preparation of Plant Extracts

Three morphotypes of E. alba plants were collected and washed under running tap water, air-dried for 4-5 days. Leaves were then homogenized separately to fine powder and stored in airtight container for further use.
Aqueous Extract

Five gram of dried plant material was extracted in 100 ml distilled water for 6 h at 60 °C. Extract was filtered and centrifuged at 6000 rpm for 25 min. The supernatant was collected and concentrated in a rotary evaporator. Dried extract was dissolved in DMSO (1:1 w/v) and stored at 4 °C in airtight bottle.

Solvent Extract

For making solvent extract 5g of dried plant material (leaves) extracted in Methanol or Acetone (100 ml) and kept on a rotary shaker for 48 h. Thereafter, it was centrifuged and filtered through Whatman’s filter paper no.1. The filtrate was concentrated in rotary evaporator. The concentrate was dried and dissolved in DMSO and stored at 4 °C.

3.6.2 Test Organisms

Bacteria used for antimicrobial activity assay were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

*Bacillus subtilis* MTCC ACC NO. 2757  
*Escherichia coli* MTCC ACC NO. 3261  
*Klebsiella pneumoniae* MTCC ACC NO. 3384  
*Pseudomonas aeruginosa* MTCC ACC NO. 1035  
*Staphylococcus aureus* MTCC ACC NO. 740/96

All bacteria were sub-cultured in nutrient broth (13g/L) at 37°C for 24 h.

3.6.3 Disc Diffusion Method

The antimicrobial activities of aqueous, acetone and methanol extracts were performed by Disc Diffusion method (Bauer et al., 1966) using *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*. All tests were carried out in triplicate. Sterilized petri dishes were preseeded with 30 ml of agar containing growth medium and 0.5 ml of inoculation (inoculum’s size $10^4$ cells/ml). Sterile paper discs measured 9 mm
diameter that absorbed 20 µl of the test sample was placed on the solidified plates under aseptic conditions. The inoculated plates were stand for 1 h and then incubated at 37±1 °C for 24 h. The diameter of inhibition zone was measured and compared with those of the standard references i.e. positive control (20 µl Chloramphenicol) and negative control (DMSO).

**Calculations:**

Activity index was calculated by comparing the zone of inhibition of the leaf extract with that of chloramphenicol.

\[
\text{Activity Index} = \frac{\text{Inhibition zone of test sample (extract)}}{\text{Inhibition zone of standard antibiotic}}
\]

3.7 **IN VITRO STUDIES**

Tissue culture techniques were used in *Eclipta alba* for micropropagation as well as for inducing genetic variability. Attempt has been made to optimize the *in vitro* culture conditions of *E. alba* for mass multiplication and callus culture studies. Three accessions of *Eclipta* germplasm representing different morphotypes i.e. erect, semierect and prostrate were selected for tissue culture studies.

3.7.1 Explants

For mass propagation, the nodal cuttings obtained from the plants of accessions EPBP1, SPBP1 and PPBP1 served as the explants while for callus studies seedlings obtained from seeds served as explants.

3.7.2 Explant Sterilization Treatment

The explants (healthy young stem nodes) with a single axillary bud of 1.0 to 1.5 cm size were used for mass multiplication. These were washed thoroughly under running tap water for 30 min followed by treatment with a solution of 2% (v/v) Teepol and 70% (v/v) ethanol for one minute and thereafter washed three to five times with autoclaved sterilized distilled water. The explants were then taken inside laminar
flow and surface-disinfected with 0.1% (w/v) aqueous HgCl₂ solution for 5-7 minutes and finally rinsed with autoclaved distilled water (five to seven changes). The node segments were then trimmed at both ends prior to inoculation on culture media. For callus cultures, the seeds were treated with very dilute detergent solution (Teepol). They were then washed under running tap water followed by sterile double distilled water and then surface sterilized by ethanol (70%) for 1 min followed by AgNO₃ (1.0%) for 10 min. The surface sterilized seeds were germinated aseptically at 25 ± 2 ºC under a 16-h diffused light (1600 lux) /8 h darkness cycle.

3.7.3 Culture Media

The culture medium proposed by Murashige and Skoog (1962) with 3% sucrose, 0.8% agar (Table 3.4) was used as basal medium. Different growth hormones i.e. 6-Benzylaminopurine (BA), 3-Indolebutyric acid (IBA), Indoleacetic acid (IAA), Kinetin (Kn), 2, 4-dichlorophenoxyacetic acid (2, 4-D), 1-Napthalene acetic acid (NAA) were added to basal medium in various combinations or singly. In all the experiments, the chemicals used were of analytical grade (HiMedia, Merk, and Sigma).

The stock solutions of major and minor salts and growth regulators were prepared and stored under refrigeration. The culture medium was prepared by adding appropriate quantities of all the components and final volume of one liter was made up with distilled water. The pH of the medium was adjusted to 5.8 by using 0.1N NaOH or 0.1N HCl. Agar was added in the ratio of 0.8% (w/v) for solidification of medium. Before autoclaving, the media was poured into washed and dried culture tubes (15x150 mm) or conical flasks (280 ml). These culture tubes or flasks were tightly capped with cotton plugs, labeled properly and autoclaved at 121 ºC for 20 minutes at 15-psi pressure and transferred to the inoculation room where they were stored under aseptic conditions.
Table 3.4: Composition of MS medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Salts</strong></td>
<td></td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>440</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>370</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
</tr>
<tr>
<td><strong>Minor Salts</strong></td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>16.9</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>8.6</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
</tr>
<tr>
<td>KI</td>
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</tr>
<tr>
<td>Na$_2$MoO$_4$.2 H$_2$O</td>
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</tr>
<tr>
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</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
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</tr>
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<td>FeSO$_4$.7H$_2$O</td>
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</tr>
<tr>
<td><strong>Organic supplements</strong></td>
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</tr>
<tr>
<td>Myo-inositol</td>
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</tr>
<tr>
<td>Nicitinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyrodoxine-HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3%</td>
</tr>
<tr>
<td>Agar</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

3.7.4 Inoculation of Explants

The explants were surface sterilized and transferred aseptically to the sterilized glass plates under the laminar flow hood where they were further trimmed with sterile scalpel to a suitable size. For nodal cultures, both basal as well as top portion of the explants and undesirable/dead portions were removed/chopped and for callus initiation, the explants were trimmed at distal ends. The prepared explants were
carefully inoculated in autoclaved culture bottles containing MS medium with different phytohormonal supplements. The explants were placed vertically on the culture medium. The cultures were kept in culture room under 16 hour light at 27±1 °C. All subsequent subcultures were done at 4 week intervals.

### 3.7.5 Nodal Cultures

Nodal segments with single disinfected node were cultured on MS basal medium containing 3% (w/v) sucrose and with 0.8% agar and supplemented with required concentration of BAP (0.5 mg/L and 1.0 mg/L) and combination of BAP (0.5 mg/L) and NAA (0.1 mg/L) for culture initiation. After approximately 8-12 days of inoculation, the axillary bud break was seen in some explants.

### 3.7.6 Callus cultures

**a) Development of Totipotent Callus**

The excised individual parts (leaf, stem, root) of 5-7 day-old seedlings (10-12mm) were cultured for initiation of callus in MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4.52µM) and combination of 2,4-dichlorophenoxyacetic acid (2,4-D; 4.52 µM) and 6-Benzyladenine (6-BA; 4.43 µM). The percentage of callus induction and morphogenetic nature of calli were observed and recorded. The cultures were kept at 25 ± 2 ºC under a 16-h diffused light (1600 lux) /8-h darkness cycle.

**b) Maintenance of independent calli**

The initiated calli from different explants were maintained on MS medium with 2,4-D (13.58 µM) and 6-BA (4.43 µM) + 0.1% ascorbic acid. Ascorbic acid was added to prevent browning of callus cells.

**c) Plant Regeneration from totipotent callus**

After 4 days, the calli were transferred to differentiation medium placed for regeneration on supplemented with different plant growth regulators as described in Table 15. After 3-4 weeks, cultures were observed for the regeneration of callus.
3.7.7 Rooting

Elongated shoots (3-5 cm) were excised from the proliferated shoot and callus cultures and transferred to bottles containing half-strength MS medium containing 3% (w/v) sucrose and 0.4% (w/v) agar and rooting hormone. The rooting hormones used in the medium were 1.0 mg/L indolebutyric acid (IBA), 1.0 mg/L Indole acetic acid (IAA), 1.0 mg/L naphthaleneacetic acid (NAA) and half strength MS medium without any hormones.

3.7.8 Acclimatization and Transplantation

After 15-21 days of plantlets on rooting media, the rooted plantlets were transferred to plastic pots for hardening prior to their transfer to the soil. The rooted plantlets were carefully taken out of the culture tubes or flasks using forceps to avoid the mechanical injury to the plantlets and washed gently under running tap water to remove any trace of media. For hardening, these plantlets were planted in small plastic pots (10 cm diameter) containing autoclaved garden soil and farmyard soil in the ratio of 1:1. This mixture was treated with antifungal agent 0.1% Bavistin solution before transplanting plantlets. The potted plantlets were covered with polyethylene sheet to maintain high humidity and were maintained inside the culture room for 15 days. The relative humidity was reduced gradually by making small holes in the polythene covering. After 30 days the plantlets were transplanted to Green House at Botanical Garden. After 10-15 days, the polythene sheets were removed and plants were regularly watered. Established plants were then transplanted to earthen pots containing garden soil under natural conditions. Initially the newly planted samplings were watered twice to prevent wilting. The morphological characteristics, growth characteristics and floral features were examined.
3.8 CULTIVATION TRIAL

Cultivation trial of *E. alba* plants was conducted to check the response of the plants to different agronomic conditions. For this experiment, the seedlings of plants obtained from seeds of the accession PPBP1 were planted in randomized block design with three replications each. Each plot was 1 m² in size and plants were grown in rows (space 33.3 cm) with plant to plant spacing of 33.3 cm. A total of 27 plots were cultivated with eight treatment combinations and one control. The field experiment in randomized block design with eight treatments and three replications was conducted at Botanic Gardens, Punjabi University, Patiala.

3.8.1 Procedure of the experimental field

Experimental field was ploughed and leveled. Field was prepared into treatment beds as per the layout plan by making borders and irrigation channels manually.

**Raising the seedlings**

For raising the seedlings, seeds were sown in the nursery bed. The soil has prepared up to a depth of 30 cm and mixed with farm yard manure, gently covered with soil and watered using a sprinkler. Seedlings are ready for transplanting when they are about 45 days old.

**Plot Treatments**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₀</td>
<td>Control</td>
</tr>
<tr>
<td>N₁</td>
<td>NPK = 2.5g/m²</td>
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<tr>
<td>N₂</td>
<td>NPK = 5g/m²</td>
</tr>
<tr>
<td>N₀ + FYM₁</td>
<td>FYM = 1kg/m²</td>
</tr>
<tr>
<td>N₁ + FYM₁</td>
<td>FYM = 1kg/m² + NPK = 2.5g/m²</td>
</tr>
<tr>
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<tr>
<td>N₁ + FYM₂</td>
<td>FYM = 2kg/m² + NPK = 2.5g/m²</td>
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<tr>
<td>N₂ + FYM₂</td>
<td>FYM = 2kg/m² + NPK = 5g/m²</td>
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The plan of layout of Experiment

Block I

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<thead>
<tr>
<th>N₀</th>
<th>N₁</th>
<th>N₂</th>
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<td>N₂</td>
<td>N₀</td>
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<tr>
<td>N₂</td>
<td>N₀</td>
<td>N₁</td>
</tr>
</tbody>
</table>

Block II

<table>
<thead>
<tr>
<th>N₁ + FYM₁</th>
<th>N₂ + FYM₁</th>
<th>N₀ + FYM₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂ + FYM₁</td>
<td>N₀ + FYM₁</td>
<td>N₁ + FYM₁</td>
</tr>
<tr>
<td>N₀ + FYM₁</td>
<td>N₁ + FYM₁</td>
<td>N₂ + FYM₁</td>
</tr>
</tbody>
</table>

Block III

<table>
<thead>
<tr>
<th>N₂ + FYM₂</th>
<th>N₀ + FYM₂</th>
<th>N₁ + FYM₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₀ + FYM₂</td>
<td>N₁ + FYM₂</td>
<td>N₂ + FYM₂</td>
</tr>
<tr>
<td>N₁ + FYM₂</td>
<td>N₂ + FYM₂</td>
<td>N₀ + FYM₂</td>
</tr>
</tbody>
</table>

3.8.2 Fertilizer application

Farmyard manure and NPK were applied at the time of planting in the plot according to the plot treatments. NPK in the ratio of 20:20:20 and 1 kg/m² and 2 kg/m² FYM is applied to block II and III.

3.8.3 Irrigation

After transplanting, irrigation was provided twice a week till one month, so that the plants establish well. Later, it was given at weekly intervals depending upon the rainfall and soil moisture status.

3.8.4 Weeding

Weeding cum hoeing was done manually and regularly before each irrigation.

3.8.5 Observations

Observations were made on *E. alba* plants after attaining 4.5 months age. Plants were evaluated for parameters i.e. plant spread, number of nodes and branches on stem, leaf size, diameter of open flower head and carbohydrate and protein contents.
3.9 STATISTICAL ANALYSIS

3.9.1 Standard deviation

Standard deviation (SD) is a measure of how widely values are dispersed from mean. Standard deviation is the square root for its variance. For various observations mean values were taken and the standard deviation of mean was calculated as follows:

\[ SD = \sqrt{\frac{n \sum x^2 - (\sum x)^2}{n(n-1)}} \]

Where, \( x \) = value of whole variable
\( n \) = total number of observations
\( SD \) = standard deviation
\( \sum \) = summation

3.9.2 Correlation Coefficient

Correlation coefficient provides the relationship between two or more set of quantitative variables. It was calculated using Karl Pearson’s method based on the following formula:

\[ r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{n \sum x^2 - (\sum x)^2} \sqrt{n \sum y^2 - (\sum y)^2}} \]

Where
\( r \) =correlation coefficient
\( n \) = number of variable x
\( x \) = one variable
\( y \) = a second variable

3.9.3 Analysis of variance (ANOVA)

This was done to compare the mean of more than two groups. F-ratio was calculated by using Statistica version 7.0 and cpcs software. One-way ANOVA was used to compare the morphological and biochemical parameters in the three morphotypes of *Eclipta alba*