CHAPTER-3 MATERIALS AND METHODS

The impact of water deficit and salinity on the morphology, physiology and biochemistry of *Oenothera biennis* L. was studied. The methodology adopted is presented in this chapter.

### 3.1 Materials

The seeds of *Oenothera biennis* L. were obtained from Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP). Experiments were conducted in the Botany laboratory and nursery area of Shoolini University of Biotechnology and Management Sciences, Solan.

### 3.2 Methods

#### 3.2.1 Seed Germination Assays

Seeds, selected for uniformity (on the basis of colour and size), were surface sterilized with 0.1% HgCl$_2$ for 5 min. followed by thorough washing under tap water. Then, the seeds were soaked in distilled water (control) or in solution of stated concentration of the effector substances namely i) PEG (5%, 10%, 15% and 20%) and ii) NaCl (25 mM, 50 mM, 75 mM and 100 mM) for 24 h at 25±2°C. Thereafter, the seeds were transferred to petriplates (100 mm×15 mm) lined with two layers of filter paper moistened with distilled water (control), PEG and NaCl solutions of same concentrations. PEG and NaCl solutions were applied initially (one time) at a rate of 5 ml/plate. Subsequently, distilled water was added, when required, to maintain the substratum wet. The seeds were allowed to germinate in an incubator at 25±2°C under continuous illumination provided by fluorescent white light (PAR: 40 μmol m$^{-2}$s$^{-1}$). Emergence of 2-5 mm radicle was taken as seed germination (ISTA, 1966). Germination was recorded regularly until the final count. Experiments were performed in triplicate; each replicate comprised 30 seeds. After 31 days of germination, seedling growth was measured in terms of root length, shoot length, seedling fresh weight and seed vigor index. The shoot and root lengths were measured with scale while seedling weight was measured by weighting balance.
Seed vigour index (SVI) determined by using the formula of Abdul-Baki and Anderson (1973):

\[ SVI = \sum \frac{(Seedling \ germination \ percentage \times Seedling \ length(cm))}{100} \]

### 3.2.2 Seedling Growth Assays

Surface sterilization of seeds of *Oenothera biennis* L. was done with 0.1% HgCl\textsubscript{2}, rinsed three times with distilled water prior to sowing. Before sowing, damaged and insect infected seeds were discarded and the empty ones were eliminated using floating method in distilled water. Seeds were sown in nursery beds (10×1m) during the month of September, 2012 in the Herbal Garden of Shoolini University, Solan (latitude 30°51’N, longitude 77°07’E and altitude 1195 m), where the average annual rainfall was 1315.6 mm. The average maximum and minimum temperatures were 32°C and 2°C, respectively. When the first three leaves appeared the seedlings were transferred to the ceramic pots of fixed capacity (25 cm diameter). The pots were filled with 7 kg uniform soil mixture containing soil: sand in 2:1 ratio. Three seedlings per pot were transplanted but after some days the seedlings were thinned to two seedlings per pot. Plants were irrigated daily with Hoagland’s solution for one month before carrying the experiment and during experiment solution was given weekly. For each treatment 60 plants were selected. Destructive and non-destructive sampling was done for analyses. For non-destructive observations pots were marked randomly within a treatment. Destructive observations were taken in triplicate at an interval of 30, 60, 90, 120, 150, 180 and 210 days. Each experiment was replicated thrice. The pots were arranged in a completely randomized design and the position of the pots was changed weekly to avoid position effect.

### 3.2.3 Induction of Water and Salinity Stress

Water-deficit and salt stress were induced after one month of transplanting the plants to pots, when the plant adapted in pots. Water-deficit experiment was conducted by gravimetric method in which initially soil water potential was maintained by tensiometer. Soil water potential was restored everyday by watering plants daily in the morning hours for seven months. For restoring soil water potential to the pots, water was added to the surface of the pots as well as to the bottom of the pots through already inserted polytubes in growing medium. The water potential was
maintained at -0.01, -0.03, -0.05 and -0.07 M Pa. For salt stress, potted plants were given 50 ml of fixed quantity of solution (25 mM, 50 mM, 75 mM and 100 mM NaCl) after one week. After an interval of 30, 60, 90, 120, 150, 180 and 210 days of applying stresses (water and salt), the plants were analysed for growth, physiological and biochemical aspects. Untreated and stress-treated plants were kept under natural photoradiation.

3.3 Physiochemical Characteristics of Soil

The physiochemical analysis of soil was done before transplanting the plants to the pots. Soil was analysed for various physico-chemical properties.

3.3.1 Soil pH

Air-dried soil (5g) was mixed in 50 ml distilled water and stirred for 1h on magnetic stirrer for uniform mixing. Adjusted pH meter for the temperature. Calibrated with two buffers, one in the acidic range and the other in the alkaline or at neutral pH. Carefully inserted the glass electrodes in the paste and pH was recorded. The average pH value was 6.76 (Table 3.1).

3.3.2 Electrical Conductivity

Transferred 20 g of soil sample in a 100 ml beaker and added 40 ml of distilled water and kept for 1 h on a shaker. Allowed to stand until clear supernatant liquid was obtained. Calibrated the conductivity bridge with the help of standard KCl solution and determined the cell constant. Determined the conductivity of the supernatant liquid with conductivity meter. The average electrical conductivity of the soil was 0.54 ds m⁻¹ (Table 3.1).

3.3.3 Estimation of Nitrogen

Soil (20 g) was weighed and placed in Kjeldahl distillation flask and added some amount of distilled water. Then 100 ml of 0.32% KMnO₄ solution and 100 ml of 2.5% NaOH solution was added and cork was immediately fixed and heated. 25 ml of N/50 H₂SO₄ was pipette out in a conical flask and 3 drops of methyl red indicator were added and the end of delivery tube was dipped into it. Ammonia gas was distilled from the distillation flask with the help of hot plate and about 30 ml of the distillate was collected. Then distillate was titrated against N/50 NaOH. The
percentage of nitrogen was determined by using formula:

\[
\text{Percentage of available nitrogen} = \frac{[(25 - x) \times 0.00028 \times 100]}{20}
\]

And available nitrogen in kg ha\(^{-1}\) was determined as

\[
\text{Available nitrogen} \left(\text{in} \frac{\text{kg}}{\text{ha}}\right) = \text{Percentage} \times 22400
\]

The available nitrogen in soil was 77.50 kg ha\(^{-1}\) given in Table 3.1.

Table 3.1: Physico-chemical characteristics of soil used in pots for growing *Oenothera biennis*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Characteristics of Soil</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH</td>
<td>6.76</td>
</tr>
<tr>
<td>2</td>
<td>Electrical conductivity (ds m(^{-1}))</td>
<td>0.54</td>
</tr>
<tr>
<td>3</td>
<td>Available Nitrogen (kg ha(^{-1}))</td>
<td>77.50</td>
</tr>
<tr>
<td>4</td>
<td>Available Potassium (kg ha(^{-1}))</td>
<td>92.97</td>
</tr>
<tr>
<td>5</td>
<td>Available Phosphorus (kg ha(^{-1}))</td>
<td>0.128</td>
</tr>
</tbody>
</table>

### 3.3.4 Estimation of Potassium

Potassium in soil was estimated by ammonium acetate method (Hanway and Heidel, 1952). Weighed 5 g of soil in 100 ml conical flask. Added 25 ml of the neutral 1N ammonium acetate solution and shaken for 5 minutes. Filtered through Whatman No. 1 filter paper. Measured K concentration in the filtrate using flame photometer. A standard curve was prepared for K\(^+\) ions between concentration and intensity and this curve was used to find the concentration of K\(^+\) ions in soil. Calculated values of “C” from standard curve. The amount of potassium was calculated (in kg ha\(^{-1}\)) using formula:

\[
K = \frac{[C \times \text{Volume of extractant} \times 2.24]}{[\text{weight of soil taken}]}
\]

The results revealed that the available potassium in soil sample was 92.97 kg ha\(^{-1}\) (Table 3.1).
3.3.5 Estimation of Phosphorus

A stock solution of 100 µg/l phosphorus was prepared by dissolving 0.439 g potassium dihydrogen orthophosphate (KH₂PO₄) in 500 ml distilled water. Then, 25 ml of 7 N H₂SO₄ was added and diluted with distilled water to make it one litre. To make standard solution 1, 2, 3, 4 and 5 ppm solution were taken from stock solution in different beakers and made final volume 10 ml by adding distilled water in each beaker. In each beaker one drop of p-nitro phenol indicator and 2.5 M H₂SO₄ was added to obtain a clear solution. This gave 0.1, 0.2, 0.3, 0.4 and 0.5 ppm solution of phosphorus. To each flask 8 ml of Murphy-Riley solution was added and volume made up to 50 ml by adding distilled water. Similarly, a blank solution was prepared with NaHCO₃, distilled water and Murphy-Riley reagent. After 15 minutes intensity of blue colour was recorded on spectrophotometer at 730 nm. The concentration of phosphate “C” in each sample was calculated from the standard curve and amount of available phosphorus is reported in Table 3.1.

\[
P \text{ (kg ha}^{-1}\text{)} = \frac{[C \times \text{volume of extractant} \times 2.24]}{[\text{volume of aliquot} \times \text{weight of soil}]}
\]

Where C is the concentration of P present in 10 ml aliquot (obtained from standard curve).

3.4 Morphological Parameters

In order to determine the status of growth at different growth stages i.e., at 30, 60, 90, 120, 150, 180 and 210 days after applying stresses, the following growth parameters were recorded in untreated (control), water-deficit and salinity stress plants.

- Plant height
- Number of leaves
- Leaf area
- Stem diameter
- Number of nodes
- Number of flowers
- Seed yield
- Root length
3.5 Biomass and Productivity

3.5.1 Fresh and Dry Weight

Fresh and dry weight (g/plant) of plant was recorded to determine the effect of abiotic stresses (water-deficit and salinity stress) at different growth stages i.e., at 30, 60, 90, 120, 150, 180 and 210 days after applying stresses.

3.5.2 Relative Water Content (RWC)

The fresh weight of top 5th and 6th leaves from each treatment was recorded. The leaves were then immersed in distilled water in beakers and left for 24 h. Thereafter, fully turgid leaves were weighed again. The leaves were dried in oven for 72 h at 70 °C, until constant weight of leaves was obtained. Relative water content (RWC) of leaves was calculated according to Wheatherley (1950).

\[
RWC = \frac{[(\text{fresh mass} - \text{dry mass})]}{\text{(saturated mass} - \text{dry mass})} \times 100
\]

3.6 Physiological Aspects

3.6.1 Membrane Stability Index (MSI)

Membrane stability index (MSI) was determined by recording the electrical conductivity of leaf leachates in double distilled water at 40 and 100°C (Sairam, 1994). Leaf samples (0.1 g) of top 5th and 6th leaves were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40°C for 30 min and another set at 100°C in boiling water bath for 15 min and their respective electric conductivity’s C_1 and C_2 were measured by conductivity meter.

\[
\text{Membrane stability index} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100
\]

3.6.2 Proline Content

Proline was measured by the method described by Bates et al. (1973). Extracted 0.5 g plant material (leaf and root) by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenate through Whatman No. 2 filter paper. Took 2 ml of filtrate in a test tube and added 2 ml of glacial acetic acid and 2
ml acid ninhydrin. Heated it in the boiling water bath for 1h. Terminated the reaction by placing the tube in ice bath. Added 4 ml toluene to the reaction mixture and stirred well for 20-30 seconds. Separated the toluene layer and warmed to room temperature. Measured the red color intensity at 520 nm. Ran a series of standards with pure proline in a similar way and prepared a standard curve. Determined the amount of proline in the test sample from the standard curve.

Expressed the proline content on fresh-weight-basis as follows:

\[ \text{\mu moles per g tissue} = \frac{\text{\mu g proline} \times \text{ml toluene/ml}}{115.5} \times \frac{5}{\text{g sample}} \]

Where 115.5 is the molecular weight of proline.

### 3.6.3 Total Amino Acids

Total amino acids were determined according to the method of Moore and Stein (1984). Weighed 500 mg of plant sample (leaf and root) and grind it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, added 5 to 10 ml of 80% ethanol. Centrifuged and saved the supernatant. Repeated the extraction twice with the residue and pooled all the supernatants. Reduced the volume if needed by evaporation and used the extract for the quantitative estimation of total free amino acids. To 0.1 ml of extract, added 1 ml of ninhydrin solution. Made up the volume to 2 ml with distilled water. Heated the tube in boiling water bath for 20 min. Added 5 ml of the dilutents and mixed the contents. After 15 min read the intensity of the purple color against a reagent blank in a colorimeter at 570 nm. The color was stable for 1h. Prepared the reagent blank as above by taking 0.1ml of 80% ethanol instead of the extract. For the preparation of standard, dissolved 50 mg leucine in 50 ml of distilled water in a volumetric flask. Took 10 ml of this stock standard and diluted to 100 ml in another flask for working standard solution. A series of volume from 0.1 to 1ml of this standard solution gave a concentration range 10 to 100 mg. Proceeded as that of the sample and read the color. Drawn a standard curve using absorbance versus concentration. Determined the concentration of the total free amino acids in the sample and expressed as percentage equivalent of leucine.
3.6.4 Protein Content

Protein was estimated by method described by Lowry et al. (1951). Weighed 500 mg of the plant sample (leaf and root) and grind well with a pestle and mortar in 5-10 ml of the buffer. Centrifuged and used the supernatant for protein estimation. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tube. Pipette out 0.1ml and 0.2 ml of the sample extract in two other test tubes. Made up the volume to 1 ml in all the test tubes. A tube with 1ml of water served as the blank. Added 5 ml of reagent C (alkaline copper solution) to each tube including the blank. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of reagent D (Folin-Ciocalteau Reagent), mixed well and incubated at room temperature in the dark for 30 min. Blue color was developed. Took the reading at 660 nm. Drawn a standard curve using BSA and calculated the amount of protein in the sample.

3.6.5 Total Carbohydrates

Total carbohydrates were determined in plant tissue described by Hedge et al. (1962). Weighed 100 mg of the sample (leaf and root) into a boiling tube. Hydrolyzed by keeping it in boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cooled at room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 ml and centrifuged at 10,000 rpm for 5 minutes. Collected the supernatant and took 0.5 and 1ml aliquots for analysis. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. Zero served as blank. Made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then, added 4 ml of anthrone reagent. Heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green color at 630 nm. Drawn a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculated the amount of carbohydrate present in the sample tube.

Amount of carbohydrate present in 100 mg of the sample = \[ \frac{\text{mg of glucose}}{\text{volume of sample}} \times 100 \]

3.7 Nutrient Analysis

Potassium and sodium in the acid-digest of plant sample (leaf and root) was determined using Flame photometer. Weighed 500 mg dried plant sample in 100 ml
conical flask. Added 10 ml of conc. HNO₃, placed funnel on the flask and kept for about 6-8 hrs or overnight at a covered place for pre-digestion. After pre-digestion when the solid sample was no more visible, added 10 ml of conc. HNO₃ and 2-3 ml HClO₄. Kept on a hot plate in acid proof chamber having fume exhaust system and heated at about 100°C for first 1 hr and then raised the temperature to 200°C. Continued digestion until the contents became colorless and only white dense fumes appeared. Reduced the acid contents to about 2-3 ml by continuing heating at the same temperature. Filtered through Whatman No. 42 filter paper into a 100 ml volumetric flask. Gave 3-4 washings of 10-15 ml portions of distilled water and made the volume to 100 ml. Measured Na⁺ and K⁺ concentrations in the filtrate using Flame photometer. Recorded the flame photometer readings for each of the working standards of Na and K after adjusting blank to zero. Drawn a standard curve by plotting the readings against Na and K readings.

3.8 Plant Antioxidant Defense System

3.8.1 Enzymatic Antioxidants

The enzymatic antioxidants analyzed in the leaves and roots of *Oenothera biennis* L. were SOD, CAT, POD, APX, GPX and GST.

3.8.1.1 Superoxide Dismutase

SOD (EC 1.15.1.1) activity was determined according to the method of Kakkar *et al.* (1984). The plant sample (leaf and root) (0.5g), was ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.
3.8.1.2 Catalase

Catalase (EC 1.11.1.6) activity was assayed following the method of Luck (1974). Homogenized plant tissue (leaf and root) in a blender with (0.067 M, pH 7.0) phosphate buffer (assay buffer diluted 10 times) at 1-4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed standing in the cold with occasional shaking and then repeating the extraction once or twice. The final volume for the assay mixture was approximately 3 ml, 240 nm wavelength read against a control cuvette containing enzyme solution as in the experimental cuvette, but containing H₂O₂-free PO₄ buffer. Pipette into the experimental cuvette 3ml H₂O₂-PO₄ buffer. Mixed in 0.01-0.04 ml sample with a glass or plastic rod flattened at one end. Noted the time required for a decrease in absorbance from 0.45 to 0.4. This value is used for calculations. One g tissue is homogenized in a total volume of 20 ml, diluted 1 to 10 volume with water and taken 0.01ml for assay. Calculated the concentration of H₂O₂ using the extinction coefficient 0.036/m mole/ml.

3.8.1.3 Peroxidase

The method proposed by Reddy et al. (1995) was adopted for assaying the activity of peroxidase. A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the plant sample (leaf and root), centrifuged and the supernatant was used for the assay. To 3.0 ml of pyrogallol solution, 0.1 ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5 ml of H₂O₂ was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

3.8.1.4 Ascorbate Peroxidase

APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada (1981). Sample (leaf and root) (0.5g) were homogenized with 5.0 ml of phosphate buffer. The homogenates were centrifuged at 5000 rpm for 10 minutes and the supernatants were used for the assay. The reaction mixture (1.5ml) contained 50 mM phosphate buffer (pH 6.0), 0.1µM EDTA, 0.5 mM Ascorbate, 1mM H₂O₂ and 50 µL enzyme extract. The reaction was started by the addition of H₂O₂ and ascorbate oxidation measured at 290 nm for1 min. Enzyme activity was quantified using the
molar extinction coefficient for ascorbate (2.8 mM \text{cm}^{-1}) and the result expressed in µmol H$_2$O$_2$ min$^{-1}$ g$^{-1}$ FW.

### 3.8.1.5 Glutathione Peroxidase

Total GPX (EC 1.11.1.7) activity was determined as described by Urbanek et al. (1991). Plant sample (leaf and root) (0.5g) were homogenized with 5.0 ml of phosphate buffer. The homogenates were centrifuged at 5000 rpm for 10 minutes and the supernatants were used for the assay. The reaction mixture (2.0 ml) contained 100 mM phosphate buffer (ph 7.0), 0.1 µM EDTA, 5.0 mM guaiacol, 15.0 mM H$_2$O$_2$ and 50 µL enzyme extract. The addition of enzyme extract started the reaction and the increase in absorbance was recorded at 470 nm for 1 min. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 mM$^{-1}$ cm$^{-1}$).

### 3.8.1.6 Glutathione-S-Transferase

Glutathione S-transferase was assessed by the method of Habig et al. (1974). Sample (leaf and root) (0.5g) were homogenized with 5.0 ml of phosphate buffer. The homogenates were centrifuged at 5000 rpm for 10 minutes and the supernatants were used for the assay. The activity of the enzyme was determined by observing the change in absorbance at 340 nm. The reaction mixture contained 0.1ml of GSH, 0.1 ml of 1-chloro-2,4-dinitrobenzene (CDNB) and phosphate buffer in a total volume of 2.9 ml. The reaction was initiated by the addition of 0.1ml of the enzyme extract. The readings were recorded every 15 seconds at 340 nm against distilled water blank for a minimum of three minutes in a spectrophotometer. The assay mixture without the extract served as the control to monitor non-specific binding of the substrates. GST activity was calculated using the extinction co-efficient of the product formed (9.6 mM$^{-1}$ cm$^{-1}$) and was expressed as nmoles of CDNB conjugated/minute.

### 3.8.2 Non-Enzymatic Antioxidants

The non-enzymatic antioxidants analyzed were ascorbic acid, carotenoids, chlorophyll, flavonoids, α-tocopherol, total phenols and lipid peroxidation.
3.8.2.1 Ascorbic Acid

Ascorbic acid was analyzed by the spectrophotometric method described by Roe and Keuther (1943). Ascorbate was extracted from 1g of the plant sample (leaf and root) using 4% TCA and the volume was made up to 10 ml with the same. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation. Standard ascorbate ranging between 0.2-1.0 ml and 0.5 ml and 1.0 ml of the supernatant were taken. The volume was made up to 2.0 ml with 4% TCA. DNPH reagent (0.5ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5 ml of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540 nm in a spectrophotometer. A standard graph was prepared using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

3.8.2.2 Carotenoids

Total carotenoids were estimated by the method described by Zakaria et al. (1979). The experiment was carried out in the dark to avoid photolysis of carotenoids once the saponification was complete. The sample (leaf and root) (0.5g) were homogenized and saponified with 2.5 ml of 12% alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was transferred to a separating funnel containing 10-15ml of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture. The final volume of the petroleum ether extract was noted. The absorbance of the yellow colour was read in a spectrophotometer at 450 nm and 503 nm using petroleum ether as blank. The amount of total carotenoids was calculated using the formula:
The total carotenoids was expressed as mg/g of the sample.

### 3.8.2.3 Chlorophyll

Chlorophyll extraction was done by using dimethyl sulphoxide (DMSO). Chlorophyll extraction technique of Hiscox & Israelstam (1979). For the extractions, glass centrifuge vials containing 7 ml DMSO were preheated to 65°C in a water bath. Chlorophyll was extracted from three disks (each 3.038 cm²; approx. 100 mg f. wt total) from each leaf sample. In preliminary trials, we found that extraction at 65°C was complete within 15–20 min and no loss of chlorophyll occurred in the heated DMSO during the first hour; the extractions were ran for 30 min. When the extractions were complete, samples were removed from the water bath and each graduated vial was topped up to 10 ml with DMSO using a Pasteur pipette; 3 ml of each extract were then transferred to disposable polystyrene cuvettes. The spectrophotometer was calibrated to zero absorbance using a blank of pure DMSO. Absorbance of both blank and sample were measured at 645 and 663 nm. The elapsed time between removal from the water bath and completion of spectrophotometer measurements was in the order of 20 min. Hiscox & Israelstam (1979) recommended the use of Arnon’s (1949) equations:

\[
\text{Chl}_a (g \text{l}^{-1}) = 0.0127 A_{663} - 0.00269 A_{645};
\]
\[
\text{Chl}_b (g \text{l}^{-1}) = 0.0229 A_{645} - 0.00468 A_{663};
\]
\[
\text{Total Chl (g l}^{-1}) = 0.0202 A_{645}+0.00802 A_{663}.
\]

The Chlorophyll (Chl) concentration of the extract calculated from these equations was then converted to leaf Chl content (mg Chl cm⁻² leaf area).

### 3.8.2.4 Tocopherol

Tocopherol was estimated in the plant samples (leaves and roots) by the Emmerie-Engel reaction as reported by Rosenberg (1992). The plant sample (2.5 g) was homogenized in 50 ml of 0.1 N sulphuric acid and allowed to stand overnight. The contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation. Into 3
stopped centrifuge tubes, 1.5 ml of plant extract, 1.5 ml of the standard and 1.5 ml of water were pipetted out separately. To all the tubes, 1.5 ml of ethanol and 1.5 ml of xylene were added, mixed well and centrifuged. Xylene (1.0 ml) layer was transferred into another stoppered tube. To each tube, 1.0 ml of dipyridyl reagent was added and mixed well. The mixture (1.5 ml) was pipetted out into a cuvette and the extinction was read at 460 nm. Ferric chloride solution (0.33 ml) was added to all the tubes and mixed well. The red colour developed was read after 15 minutes at 520 nm in a spectrophotometer.

The concentration of tocopherol in the sample was calculated using the formula,

\[
\text{Tocopherol (\mu g)} = \frac{\text{Sample } A_{520} - A_{460}}{\text{Standard } A_{520}} \times 0.29 \times 0.15
\]

### 3.8.2.5 Phenols

Phenol content was estimated by Malick and Singh (1980). Weighed 0.5 g of the sample (leaf and root) and grind it with a pestle and mortar in 10-time volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 min. Saved the supernatant. Re-extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Evaporated the supernatant to dryness. Dissolved the residue in a known volume of distilled water (5 ml). Pipette out different aliquots (0.2 to 2 ml) into test tubes. Made up the volume in each tube to 3 ml with water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 min, added 2 ml of 20% \( \text{Na}_2\text{CO}_3 \) solution to each tube. Mixed thoroughly. Placed the tubes in a boiling water bath for one minute, cooled and measured the absorbance at 650 nm against a reagent blank. Prepared a standard curve using different concentrations of gallic acid. From the standard curve the concentration of phenols in the test sample was determined and expressed as mg phenols/100g material.

### 3.8.2.6 Lipid Peroxidation

The extent of lipid peroxidation was estimated according to the method of Dhindsa et al. (1981). Lipid peroxidation was estimated from the accumulated malondialdehyde (MDA) following the method given by Dhindsa et al. (1981). In brief, the plant tissue (approx. 200 mg) (leaf and root) was homogenised with 0.1% trichloroacetic acid (TCA) (2 ml). The homogenate was centrifuged at 10,000 rpm for
10 min. and supernatant collected. The supernatant (2 ml) was reacted with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was then heated at 95°C for 45 min. and rapidly cooled in an ice bath for 5 min. Absorbance was read at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. MDA contents were determined using the extinction coefficient of 155 mM⁻¹ cm⁻¹ (Heath and Packer, 1968).

3.9 Evaluation of Radical Scavenging Effects

3.9.1 Evaluation of DPPH

The scavenging ability of the natural antioxidants of the leaves towards the stable free radical (1,1-diphenyl-2-picrylhydrazyl) DPPH was measured by the method of Mensor et al. (2001). The leaf extracts (20 µl) were added to 0.5 ml of methanolic solution of DPPH and 0.48 ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the leaf extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518 nm in a spectrophotometer. The radical scavenging activity was calculated as follows:

\[
\text{Scavenging activity} \% = 100 - \frac{A_{518}(\text{sample}) - A_{518}(\text{blank})}{A_{518}(\text{blank})} \times 100
\]

3.9.2 Evaluation of ABTS

The antioxidant effect of the leaf extracts was studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method of Shirwaikar et al. (2006). ABTS radical cations (ABTS⁺) were produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5 ml) of the three different extracts were added to 0.3 ml of ABTS solution and the final volume was made up to 1 ml with ethanol. The absorbance was read at 745 nm in a spectrophotometer and the per cent inhibition was calculated using the formula:

\[
\text{Inhibition (％) } = \frac{(\text{Control} - \text{test}) \times 100}{\text{Control}}
\]
3.10.1 Estimation of Gallic Acid

Gallic acid estimation from roots of *Oenothera biennis* L. was done by the method of Inoue and Hagerman (1988). Roots of different treatments were collected, washed, shade dried and macerated to make the powder. Ten ml of acetone (70%) was added to 100 mg of the powder and then suspended in an ultrasonic water bath for 20 min at room temperature. The suspension was centrifuged for 10 minutes at 3000 rpm at 4 °C. The supernatant was diluted with methanol to make the final volume 10 ml and kept in ice (supernatant A). Each plant was extracted twice, 200 µL of the supernatant was transferred to the test tubes (4 tubes /sample) and dried under vacuum pressure to which 600 µL of sulphuric acid (0.2 N) was added. To the three tubes 900 µL of rhodanine (0.677 %) solution was added and to the fourth tube 900 µL of methanol was added. The fourth tube was set as a blank. After 9 min 600 µL of potassium hydroxide (0.5 N) solution was added to all of the tubes. Six minutes later 12.9 ml of distilled water was added to all the tubes. After 25 minutes, absorbance of the red-purple solution was measured at 520 nm against the blank. In order to quantify the amount of free gallic acid, its calibration curve was prepared.

3.10.2: Oil Yield

Plants were harvested during maturity and seed collected. Seeds were freed of foreign materials and dirt, by winnowing. Cleaned seeds were powdered by grinding in a milling machine. From each treatment 20 g of seeds were ground and then extracted with petroleum ether in a soxhlet apparatus for 6 h (AOAC,1960).

Oil from solid material was extracted by repeated washing (percolation) with an organic solvent, petroleum ether. The powdered seeds were kept in a muslin cloth placed in a thimble of Soxhlet apparatus. The whole set up was heated in a heating mantle at 70°C. The solvent evaporated and moved to the condenser where it was converted into liquid that trickles into the extraction chamber containing the sample. The extraction chamber was made in such a way that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. The flask containing solvent and oil was removed at the end of the extraction process. The solvent in the flask was evaporated in an oven and weighed to constant value. The percentage of the oil in the sample was calculated as:
\[
\% \text{ Oil Yield (w/w)} = \frac{\text{weight in gram of extracted oil}}{\text{weight of oil seed power sample in gram}} \times 100
\]

### 3.11 Statistical Analysis

The data was analysed statistically. Mean values were calculated from measurements of six replicates and the standard error of means were determined. One-way and two-way analysis of variance (ANOVA) was applied to determine the significance of results between different treatments and Tukey’s multiple comparison test and Bonferroni’s post tests were performed at the significance level of P<0.05. In order to verify statistically the significance level in different treatments, null hypothesis (H₀) was postulated that there is no significant difference due to the treatments and assumed that there is no significant difference in parameter studied (e.g., germination %, stem length, etc.) between treatments.