Chapter-3 MATERIALS AND METHODS

The present investigations were carried out on the two medicinally important herbs i.e. *Ocimum sanctum* and *Mentha piperita* to assess their relative sensitivity to different concentrations of simulated acid rain. *Ocimum sanctum* is a biennial shrub with erect, quadrangular, branched and green stem, belonging to the family Lamiaceae. The plant has been served by the people of India for its multifarious uses since vedic times. The essential oil of *Ocimum sanctum* has about 71 percent eugenol and is comparable to that of clove oil. Eugenol is widely used in perfumery, cosmetics, pharmaceuticals and confectionary industries. The juice of the leaves is used in diaphoretic, antiseptic, antiperiodic, expectorant, antipyretic and memory improving properties. The seeds are used as remedy for urinogenital troubles. The plants are used as an antidote for snake bite. It is one among the few plants, which purifies the atmosphere.

*Mentha piperita* is a perennial glaborous herb with erect purplish or green stem. The herb is considered aromatic stimulant, stomachic and carminative and used for alloying nausea, flatulence and vomiting. Bruised leaves are used for relieving local pains and headach. It is the source of peppermint oil, extensively exploited for flavouring as well as for pharmaceutical purpose.

The different cultivars used for present studies are as follows:-

1. *Ocimum sanctum* L.cv. IC -75730
2. *Ocimum sanctum* L. cv. Local
Seeds of Ocimum sanctum L. cv. IC-75730 were obtained from NBGCR (National Bureau of Plant Genetic Resources) I.A.R.I. (Campus), New Delhi. The local seeds of Ocimum sanctum were purchased from local shop of Meerut. Explant of Mentha piperita were obtained from a farmer of District Jyotibafule Nagar. Two cultivars i.e. Kaushik & Himalaya were used for the present studies. The effects of simulated acid rain with pH ranging from 5.5, 4.5, 3.5 and 2.5 were studied on seed germination, seedling growth, crop yield and bio-chemical attributes.

**Preparation of simulated acid solutions -**

Simulated acidic solutions were prepared according to Lee et al. (1981). The solutions of different pH 5.5, 4.5, 3.5 & 2.5 were made with a combination of sulphuric acid (H₂SO₄) and Nitric acid (HNO₃) (70 m Eq. SO₄⁻², 30m Eq. NO₃). These pH values were verified using a pH meter with a glass combination electrode. The solution of 5.5 was taken as control. The pH was adjusted with the help of NaOH solution.

The experiment was divided into two i.e. (A) Long terms exposure experiments and (B) Short terms exposure experiments.

**(A) LONG TERM EXPOSURE EXPERIMENTS**

For long terms exposures, experimental seeds and explants were sown in the experimental plot of the Department of Botany, S.G.P.G. College, Saroorpur Khurd (Meerut). The seeds and explants of two different species of Ocimum sanctum and Mentha piperita were sown in the month of March 2000-2001 and 2002-2003.

The whole experiment was conducted in four sets. Twenty five days old seedlings of almost similar growth were exposed to acid solutions of different pH for three times a week. The spray was carried out with the help of one litre hand plant sprayer with a plastic spray nozzle. During the course of the experiment, plants were watered on alternate day. Out of the four, three sets were sprayed with acid water solutions of pH 4.5, 3.5 and 2.5 while the fourth set which served
as a control was sprayed with the solution of pH 5.5, nearly equal to the pH of ambient rain up to the maturity of the crops.

To study the effects of simulated acid rain, the plant samples were analysed for the following growth parameters at different intervals:

1. Length of main root and main stem.
2. Fresh weight of plant (shoot and roots).
3. Dry weight of plant (shoot and roots).
4. Number of leaves per plant.
5. Number of roots per plant.
6. Number of primary branches per plant.
7. Number of rhizomes per plant.
8. Average of primary roots length.
9. Average of primary branches length.
10. Shoot Weight Ratio (SWR) for dry weight.
11. Root Weight Ratio (RWR) for dry weight.
12. Shoot Root Ratio (SSR) for dry weight.
13. Growth Index (GI) and Tolerance Index (TI)
14. Net Primary Productivity (NPP)
15. Phytotoxicity Per cent (shoot & root) (PP)
16. Tolerance Index (TI)
17. Response Coefficient (RC)
18. Relative Water Content (RWC)
19. Number of flowers per plant.

Observations were also taken for the following yield parameters:

1. Number of spikes per plant.
2. Length of spike per plant.
3. Fresh and dry weight of spike per plant.
4. Weight of seeds per plant.
5. Number of seeds per plant.
6. Harvest index.

The Biochemical parameters were:
1. Chlorophyll contents - chl a, chl b and total chlorophyll.
2. Ascorbic acid contents
3. Phosphorus content
4. Carbohydrate contents
5. Sulphur content

To study the growth response of Ocimum sanctum and Mentha piperita cultivars, treated with various concentration of simulated acid rain, ten plants from each treatment were used for analysis. Each data is an average of three repetitions. The intact plants were gently pulled out from the soil and thoroughly washed under running tap water to remove soil particles adhering to the roots.

The parameters were recorded as follows:

**Length of main shoot and root:** The length of shoots and roots were measured in cms.

**Fresh and dry weight of shoot and roots:** After proper washing with water, the plants were dried on filter paper to remove excess water adhering to their surfaces. Then the shoot and roots were weighted separately for their fresh weights. For dry weight estimation, these plants were wrapped in separate sheets of paper, dried in an oven at 80°C for 24 hours and weighted.
**Number of leaves per plant:** Simple, opposite, decussate and exstipulate leaves with a size more than 1.5 x 0.5cms., were counted. Further senescent leaves (complete yellow or very close for shedding) were also not considered.

**Number of roots per plant:** Primary roots were counted, less than 0.5 cms. size were not taken into the consideration.

**Number of primary branches per plant:** Branches usually arise from the lower and intermediate nodes, rarely from cotyledonary or unifoliate nodes. Only the main branches, which arise from main axis were taken into consideration.

**Study with rhizome:** Rhizome study was started on 50 days old plants for their number and length. The length of rhizome was measured in cm. with the size more than 0.5 cm. was considered.

**Number of roots per plant:** Primary roots of more than 0.5 in size were considered for measurement.

**Shoot Weight Ratio (SWR), Root Weight Ratio (RWR) and Shoot Root Ratio (SRR):** Values of these attributes were related to the dry weight and biomass of plant by using following formulae –

\[
SWR = \frac{\text{Dry weight of shoot}}{\text{Dry weight of whole plant}}
\]

\[
RWR = \frac{\text{Dry weight of roots}}{\text{Dry weight of whole plant}}
\]

\[
SRR = \frac{\text{Dry weight of shoot}}{\text{Dry weight of roots}}
\]
**Growth and Tolerance Index:** The values of these attributes were correlated with the seedling height. The plants with lowest shoot and root length had least growth and tolerance index in comparison to highest shoot and root lengths. The values higher than one (>1) show the stimulating effect of the treatment while the value lower than one (<1) show inhibiting effect of the treatment. The values were calculated by following formulae -

\[
Growth\ Index\ (GI) = \frac{Growth\ in\ presence\ of\ pollutants}{Growth\ in\ controls}
\]

\[
Tolerance\ Index\ (TI) = \frac{Mean\ root/shoot\ length\ in\ treatment}{Mean\ root/shoot\ length\ in\ control}
\]

\[
Net\ Primary\ Productivity\ (NPP) = \frac{Dry\ weight\ of\ whole\ plant}{plant\ age}
\]

**Phytotoxicity Percentage (PP):** Phytotoxicity percentage was calculated for assessing the effect of simulated acid rain treatment in relation to control. The negative values of phytotoxicity percentage indicate the stimulatory effect while the positive values indicate the inhibitory effect of the treatment. It was determined by the formula of following Chou and Mullar (1972) as follows:

\[
Phytotoxicity\ Percentage\ (PP) = \frac{Root/Shoot\ length\ of\ controls\ plant – Root/Shoot\ length\ in\ treated\ plant}{Root/Shoot\ of\ controls\ plant} \times 100
\]

**Response Coefficient (RC):** Values of RC were calculated both for root and shoot for assessing the effect of simulated acid rain treatment in relation to control. The positive value of RC indicates simulation while negative values indicate retardation. RC was calculated by following formula-

\[
Response\ Coefficient\ (RC) = \frac{Value\ of\ treated\ plants – value\ of\ treated\ controls\ plant}{Value\ of\ controls\ plants}
\]

**Relative Water Contents (RWC):** This value is an assessment of the amount of water present in the plant tissues in comparison to the controls. The values of RWC were recorded by using the following formula.
Relative Water Contents (RWC) = \frac{\text{Amount in water treatment plants}}{\text{Amount in water in controls plants}}

**Number of flowers per plants:** The plan of formation of the flowers is uniform. The fully opened flowers were considered while yellow and closed flowers were not counted.

**Harvest index:** The harvest index was taken by the following formula:

\[ \text{Harvest Index (HI)} = \frac{\text{Weight of seeds per plant}}{\text{Phytomass of the plant}} \]

**Study with spikes:** Spikes studies were started on 80 days old plants for their number, length and weight. Spikes were counted till at the maturity of the crops. The length of spike was measured in cm. with the size more than 0.5 cm was considered. Fresh weights of spikes were estimated, after proper washing with water, the plants were dried on filter paper to remove acid water adhering to their surfaces. Then the spikes were weighted separate from the plants for their fresh weight. These spikes were wrapped in separate sheets of paper, dried in an oven at 80°C for 24 hours and weighted. After threshing of spikes, separately seeds were weighted. In case of *Mentha piperita* very minimum seed were formed so these seed were counted also.

**Biochemical Parameters:** These were studied as follows:

**Chlorophyll contents:** To resolve chlorophyll a, b and total chlorophyll in the leaves, 100 mg of fresh leaves were homogenised with 80% acetone with the help of sodium bicarbonate. This homogenate was centrifuged at 3000 rpm for ten minutes and a final volume of supernatant was made to 10 ml with 80% acetone. The absorbance was recorded at 663 and 645 nm by standard spectrophotometer and the amount of chlorophyll a, b and total chlorophyll was measured according to the following formulae (Arnon, 1949).

\[ \text{chl a, chl b and total obtained respectively from} \]

\[ \text{Chlorophyll a (chl a)} = 12.72 A_{663} - 2.69 A_{645} \]
Ascorbic acid contents: To determine ascorbic acid content, 200 mg of fresh leaves were homogenised in 4% oxalic acid. The homogenate was centrifuged at 3000 rpm for 10 minutes and a final volume of supernatant was made to 5 ml with 4% oxalic acid. After that 2 ml of homogenate was mixed with 5 ml of dichlorophenol indophenol (50 mg DCPIP in 150 ml distilled water mixed with 4 mg sodium bicarbonate and final volume upto 200 ml) and was mixed with 5 ml of amyl alcohol. It was shaken well in a separating funnel. After 10 minutes a pink colour was obtained in upper side. The optical density of its solution was measured at 480 nm wavelengths on a spectrophotometer.

Carbohydrate contents: Carbohydrate contents of the leaves were determined as Yemm and Wills (1954). The leaves were dried in an oven at 80°C for 24 hours and powdered in a mechanical grinder. A sample of 50 mg was digested in 10 ml of 6 NH₄Cl for 3 hours, and then dissolved in sodium carbonate, when the precipitates do not finish. After centrifuging the digestive material for 10 minutes at 3000 rpm and the volume was made 20 ml with double distilled water. Out of it, 0.1 to 0.5 ml of the filtered digested material was taken and 3 ml of anthrone reagent (0.2% anthrone dissolved in conc. H₂SO₄) was added to it. The solution was rapidly mixed, made upto 5 ml by adding distilled water and placed in an icebath for 10 minutes to cool and develop blue green colour. The optical density of blue green solution was taken at 630 nm on a standard spectrophotometer. The amount of carbohydrate in the solution was determined with the help of calibration curve prepared by using various dilutions of glucose solutions.

Phosphorus content: Phosphorus content of leaves were determined as per Alen, et al. (1954). The leaves were dried in an oven at 80°C for 24 hours and powdered in mechanical grinder. A sample of 50 mg was homoginised with 10 ml NaHCO₃ (42.0 gm/ 1 litre) and 1 pinch of activated charcoal and allowed to stand on shaker for 30 minutes. Five ml filtrate of the solution was taken and added 2.5 ml molybdate reagent (15 gm ammonium molybdate dissolved 300 ml distilled water and 1 ml H₂SO₄)
water and add 348 ml conc. HCl and add distilled water to make volume 1000 ml) and 5 ml distilled water and swirled after that added 1 ml working stannous chloride solution and make the volume 12.5 ml with distilled water. The optical density of yellow solution was taken at 660 nm on a standard spectrophotometer. The amount of phosphorus contents in the solution was determined with the help of calibration curve.

**Sulphur content**: The quantity of sulphur in the leaves of treated plants was determined by colorimetric procedure as described by Palaskar et al. (1981). The sulphur is extracted in 15% CaCl₂·2H₂O solution in 1:5 ratio of leaves to solution using 50 gm of leaves and 30 minutes shaking time (Williams and Steinbergs, 1959).

Five ml of barium chromate solution is pipetted into 100 ml volumetric flask to which 1.2 ml of 5N ammonium hydroxide is added in order to reduce the free acidity to about 0.05N. After shaking and standing the extract of leaves for about half an hour 1 ml of ammonium hydroxide is added to precipitate the un-reacted barium chromate. The volume is made up to 100 ml with distilled water. The flask is stopper and inverted 2-3 times. The content is filtered through dry whatman No. 42 filter paper rejecting the first few ml of filtrate. The intensity of yellow colour in the filtrate is measured in spectrophotometer using deep bull (420 mu) filter and the concentration found out from the standard curve. Standard curve prepared by using 10 ppm working solution of sulphur.

**Short Term Exposure Experiments**

**Seeds and explants germination trials**: For germination trial uniform healthy and viable seeds of *Ocimum sanctum* and explants of *Mentha piperita* rhizome of 2.5 cm length assumed as seeds were sterilised with 0.0001/M mercuric chloride solution for 2 minutes. One hundred seeds or explants in each set, were pre-soaked with acid solution of pH-5.5, 4.5, 3.5 and 2.5 and were allowed to germination in petridishes (10 cm in diameter). Each petridish has only five seeds or explants lined with Whatman no. 40 filter paper dishes, associated with an underline of a thin uniform pad of sterilized cotton. Sets of each cultivar were
arranged equidistantly in concentrate rings over the filter paper. Ten ml of simulated acid rain solution of pH 5.5, 4.5, 3.5 and 2.5 was gently our over each set.

The emergence of radical to 1-2 mm length was considered as successful germination and germinated seeds were counted every day. The process was continued until no further germination was noticed. From the data thus obtained germination percentage, seed vigour and mean germination frequency, seed germination index were calculated by following formula given by Agarwal (1980) as follows:

\[
\text{Quotient } (Q) = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}}
\]

\[
\text{Seed Vigour Percentage} = \frac{\text{Quotients of daily counts}}{\text{Number of days of germination}}
\]

Mean Germination Frequency (MGF) (It is a rate of seed germination)

\[
\text{MGF} = \frac{\text{Maximum number of seeds germination}}{\text{Period in which maximum germination achieved}}
\]

**Statistical treatment of the results:** The different quantitative measurements obtained with different treatments are statistically analysed. For verifying null hypothesis, ANOVA test is applied. The significant values were further computed to obtain critical difference at 5% and 1% level of significance that revealed the difference between any two treatments.