Chapter II

MATERIALS AND METHODS

2.1 Material :

Euphorbia geniculata Orteg. a member of family euphorbiaceae is worked out for its eco-physiology during present investigation. It is commonly found as a weed in gardens, cultivated fields, under tree shade, moist and waste watered places and elsewhere throughout the year.

For field studies, morphological and phytosociological observations of E. geniculata were done in the natural population in the campus of Yeahwantrao Chavan College of Science, Karad. For the study of seed attributes, mature seeds are collected from uniform populations of E. geniculata.

Leaf architecture sdtudy was done in fresh, alternate and opposite leaves of the plant at flowering stage. Entire plants of E. geniculata along with soil at flowering stage were brought to the laboratory and analysed for the study of nitrogen metabolism.

Seasonal variations in physico-chemical constituents was worked out by using entire plant of E. geniculata. Plant samples were collected throughout continuous twelve months of the year and at fifteenth date of each month.

Stomatal behaviour in E. geniculata was studied in the natural habit at Shivaji University campus, Kolhapur. For pathophysiological studies healthy and infected plants at flowering stage, from uniform populations were collected.

Every time at least two replicates were maintained and maximum care was taken to minimise personal error.

2.2 Methods :

2.2.1 General information :

Data regarding the geographical distribution pattern of the E. geniculata in India and the world was obtained through various circles of Botanical survey of India and Kew herbarium.

2.2.2 Field studies :

i) Habitat : Habitat of E. geniculata was studied directly in the field, with reference to its edaphic and climatic conditions.

ii) Morphology : Pollination and Dispersal : Morphology of the plant was studied with the help of laboratory equipments and Flora of the Presidency of Bombay (Cooke, 1906). Pollination mechanism was observed according to the observations made by Reddi and Reddi (1985); while dispersal mechanism was studied in the light of report of Zohary (1937).

iii) Phenology and Life cycle : Phenological observations and life cycle study with respect to its seedling emergence, vegetative period, initiation of flowering and fruit setting was studied according to the method described by Misra (1968).

iv) Growth performance : Growth performance of E. geniculata under different habitats like drought and moist, full light and tree shade was studied according to the method of Misra (1968) and Yadav (1983). The species under selected sites were analysed for its morphological parameters like length and basal diameter of root and stem, root:shoot ratio, number of interrnodes, number of branches, number of alternate and opposite leaves, length, breadth and area of leaf, total leaves per plant, seed output per plant and reproductive capacity of plant etc.

v) Phytosociological studies : Phytosociological study at different habitats was carried out by quadrat method . A list quadrat of one square meter was used. At each study site atleast ten quadrats were studied at random. The plant species present in each quadrat were noted and total number of individuals of each species was counted. The values of abundance, density, percentage frequency were calculated by using the formulae given by Misra (1968) and Ambasht (1984).

$$\text{Abundance} = \frac{\text{Total no. of indivuduals of a sps. in all quadrats}}{\text{Number of quadrats of occurrence of the sps.}}$$

$$\text{Density} = \frac{\text{Total no. of individuals of a sps. in all quadrats}}{\text{Total number of quadrats studied}}$$

$$\text{Percentage frequency} = \frac{\text{Total number of quadrats of occurence}}{\text{Total number of quadrats studied}} \times 100$$

2.2.3 Seed attributes :

Initially the seeds of about fifty plants were collected randomly and separately from their natural habitats. The seeds were air dried for fifteen to twenty days at laboratory conditions and stored separately in dry glass stoppered bottles.

Seed attributes were studied with respect to, seed output per plant, colour, shape, diamension, seed index, air dry weight, oven dry weight, moisture content, viability, germination, dormancy, reproductive capacity and ecological amplitude etc.

i) Seed output per plant :

Seed output per plant was calculated by using the formula,

Seed output per plant = Seeds/capsule x capsules/plant

Seed per plant is an average of minimum fifty plants.

ii) Seed diamension and seed index :

Length and breadth of seed was measured by "Micrometer Screw gauge" manufactured by Paramount Scientific Works India. The seed index was calculated by using the formula (Yadav, 1983).

Seed index = Length x Breadth

Seed index is an average of minimum thirty seeds.

iii) Air dry weight and Oven dry weight :

Air dry weight and oven dry weight per hundred seeds is an average of ten to five lots, each of hundred seeds. The weight of seed was determined to the accuracy of 0.001 g using chemical balance.

iv) Moisture content :

Percentage moisture content of air dry seed was measured according to method described by Zeleny (1961) and was determined by using formula -

Percentage moisture content of air dry seed =

$$= \frac{\text{wt/100 air dryseeds - wt/ 100 oven dry seeds}}{\text{wt/100 air dry seeds}} \times 100$$

v) Viability :

Average percentage of viable and non viable seeds per plant was determined by Topographical Tetrazolium or TZ Test as described by Lakon (1949). For each treatment three replicates of twenty seeds each were used and mean values of viable and nonviable seeds were obtained.

vi) Germination study :

Air dried seeds have been used for all germination tests unless and otherwise mentioned. The percentage germination is expressed on the basis of

the number of seedlings emerged out from total number of seeds tried for germination. Protrusion of radicle through seed coat is considered as an index of germination.

Germination experiments were made in Petriplates (7-10 cms. in diameter) on a single or inbetween two ordinary blotting papers. The seeds were surface sterilized with 10% calcium hypochlorite for ten minutes and then well washed in distilled water before sowing. For each treatment three replicates of twenty seeds each were used and mean values of germination percentage were obtained

(a) Dormancy test :

To test dormancy, freshly harvested seeds were subjected to germination test as described above.

(b) Effect of air dry storage on germination :

To observe effect of storage on germination, seed samples ranging from zero day storage to fourteen days air dry storage were kept for germination. The effect of storage period on germination was observed in terms of percentage germination.

(c) Seed vigor :

Seed vigor was measured in terms of speed of germination as described by Kozlowski (1972). Air dry seeds were kept for germination and germination percentage was recorded after every four hours upto fifty six hours.

vii) Reproduction capacity :

Reproductive capacity of E. geniculata under drought and moist habitats was calculated by usding the formula of Salisbury (1942).

$$\text{Reproductive capacity} = \frac{\text{Average seed output} \times \text{Average \% germination}}{100}$$

viii) Effect of water on seed coat :

Air dry seeds were placed in petriplate containing water and observed under binocular dissecting microscope for interval of twenty minutes. The changes induced by water on helices embedded in seed coat were noted according to the observations of Jordan et al.,(1985). The helices on seed coat were tested for its chemical nature with the help of Acid-Schiff test and I_2KI.

iv) Ecological amplitude of the seed :

(a) Longevity of seed :

To find out the longevity of seeds, air dry seeds of 6 months, 12, 18, 24 and 30 months were tested for its viability by TZ method as described earlier.

(b) Temperature :

To study ecological amplitude of seed with respect to temperature, air dry seeds were subjected to various temperature treatments from $40^{o}C$ to $80^{o}C$ and tested for its viability by TZ method.

2.2.4 Leaf architecture and organic constituents :

The physiological characteristics associated with pathways of photosynthesdis such as leaf architecture and organic constituents were studied in fresh leaves of E. geniculata.

i) Leaf architecture :

External morphology of leaf was studied by usual routine method. For anatomical studies thin transdver hand sections were taken. To remove chlorophylls the sections were boiled in alcohol for few minutes and observed uncer light microscope after staining it with dilute I_2KI.

ii) Organic constituents :

For estimation of organic constituents like moisture content. TAN, chloro phylls and mesophyll succulence, mature and fully expanded fresh leaves were used. Collected leaves were washed with distilled water and blotted to dry and used for analysis.

(a) moisture content ;

Leaf moisture content was determined as described by Patil (1980). The leaves were dried in oven adjusted to 80°C temperature until constant. Moisture percentage was calculated by using the formula,

$$\% \text{ moisture} = \frac{\text{Fresh wt. - Dry wt.}}{\text{Fresh wt.}} \times 100$$

(b) Titratable acid number (TAN) :

Tan was estimated by method of Thomas and Beevers (1949). The leaves were first washed with distilled water and then blotted to dry. 2 g of sample was accurately weighed and boiled for half an hour in distilled water. It was cooled and filtered through the double layered musline cloth. The volume of filtrate so obtained was recorded. 10 ml. of this extract was titrated against N/40 NaOH, using phenolphthalein as an indicator. N/40 NaOH was standardized with N/40 oxalic acid using phenolphthalein as an indicator.

Titratable acid number (TAN) represents the number of ml. of decinormal NaOH required to neutralize the acid contents in 100 g of fresh tissue and was calculated by the following formula,

$$\text{TAN} = \frac{\text{Ml. of oxalic acid taken for titration}}{\text{Burette reading for 10 ml. oxalic acid}} \times \frac{\text{Extract reading}}{\text{ml.of extract taken for titration}} \times \frac{\text{Volume of extract}}{\text{wt. of plant material}} \times \frac{100}{4}$$

(c) Chlorophylls :

The chlorophylls were estimated by the method of Arnon (1949). The leaves were washed thoroughly with distilled water and blotted to dry. 0.5 g of the leaves were accurately weighed and homogenized in mortor with pestle. Chlorophylls were extracted in 80% acetone (about 40 ml.), then the extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue was washed thoroughly with 80% acetone collecting the washings in the same container. Finally the volume was made to 100 ml. with 80% acetone All the operatioins were done in dim light, and at the lowest possible temperature. Optical density of the filtrate was read at 645 and 663 nm.

Chlorophylls (mg/100 g fresh tissue) were calculated by using the following formulae,

Chlorophyll 'a' = (12.7 x A.663) - (2.69 x A.645) = X

Chlorophyll 'b' = (22.9 x A.645) - (4.68 x A.663) = Y

Chlorophyll 'a+b' = (8.02 x A.663) + (20.20 x A.645) = Z

$$\text{Chlorophyll a/b/a+b mg/100 g fresh tissue} = \frac{x/y/z \times \text{vol. of extract} \times 100}{100 \times \text{wt. of plant material (g)}}$$

$$\text{Chlorophyll a:b ratio} = \frac{\text{mg of chlorophyll 'a' / 100 g}}{\text{mg of chlorophyll 'b'/100 g}}$$

(d) Mesophyll succulence :

Mesophyll succulence of leaf was calculated by using the formula as described by Karadge (1981).

$$\text{Sm (mesophyll succulence)} = \frac{\text{Moisture percentage of leaf}}{\text{Total chlorophylls}}$$

2.2.5 Stomatal studies :

Stomatal studies were done by the procedure described by Waghmode and Joshi (1979). The impressions of upper and lower epidermis of alternate and opposite leaves were obtained with the help of nail paint. These imprints were used to determine stomatal frequency. Stomatal frequency was calculated by counting the number of stomata per one square milimeter.

Diurnal variatioins in stomatal behaviour of opposite and alternate leaves of E. geniculata was studied in natural habit at Shivaji University Campus, Kolhapur.

Stomatal behaviour with respect to relative humidity, light quantum, leaf temperature, diffusive resistance, transpiration rate was observed from both the epidermis of alternate and opposite leaves, with the help of Steady State Autoporometer (LI cor in., Model LI-1600, of USA).

2.2.6 Nitrogen metabolism :

To study nitrogen metabolism with respect to total nitrogen, total proteins and enzymes, fresh and oven dry material of E. geniculata was used. From oven dried root, stem and leaves total nitrogen and total proteins were estimated. While fresh root, stem and leaves were used to study activity of enzyme nitrate reductase and nitrite reductase.

i) Total nitrogen :

The total nitrogen content in the oven dried root, stem and leaves were estimated, according to the method of Hawk et al., (1948). 0.5 g of oven dried material was digested in Kjeldahl flask with sulphuric acid (1:1 dilution) and a pinch of microsalts (mixture of anhydrous copper sulphate and potassium

sulphate in the proportion of 1:40) till a colourless liquid was obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to the volumetric flask and volume was adjusted to 100 ml with distilled water. Then it was filtered on next day through dry filter paper. The filtrate was used for estimation of nitrogen.

Two ml. of this filtrate was taken in Nesslor's tube (35 and 50 ml marked). In other tubes different concentrations of standard ammonium sulphate (0.05 mg nitrogen / ml.) were taken. One tube was kept as a blank without ammonium sulphate. To each of these tubes was added a drop of 8% potassium bisulphate. The volume of all these tubes was adjusted to 35 ml. with distilled water. 15 ml of Nessler's reagent was then added in each tube. Nessler's reagent is a mixture of reagent A (7 g KI and 10 g HgI_2 dissolved in 40 ml distilled water) and B (10 g NaOH dissolved in 50 ml. distilled water) in the proportion of 4:5. The colour intensity of the orange brown product ($NH_4Hg_2I_3$) produced by the reaction between NH_3 liberated from the sample and reagent, was measured at 520 nm on Toshniwal's VIS - Spectrophotometer. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

ii) Total proteins :

To estimate total proteins in the sample, similar method for estimation of total nitrogen was used and total proteins were determined by using the formula, as described by Gharge (1984).

Total proteins = Total nitrogen x 5.7 (factor)

iii) Enzymes of Nitrogen Metabolism :

(a) Nitrate reductase (E.C. 1.6.6.2) :

To determine activity of enzyme nitrate reductase (NR) in fresh root, stem and leaves of E. geniculata Orteg, in vivo method of Jaworski (1971) as described by Knyl (1974) was followed with slight modifications.

Leaf discs of 1 cm. diameter were cut with the help of leaf punch, while stem and root were cut into small pieces of uniform size. 500 mg of plant tissue was suspended in 10 ml of a standard incubation medium containing phosphate buffer (pH 6.2), 20 mM KNO_3, 5% (v/v) n-propanol and 1.25% Triton x - 100 in sealed jars and incubated in the dark for 30 minutes in case of leaves, while root and stem samples were incubated in the dark for 60 minutes Nitrate reductase activity was measured by NO_2^- production which was detected by treating 0.4 ml of 1% sulfanilamide in 2 N. HCl and 0.3 ml of 0.02% NEEDA (N-1 Naphthylethylene diamide hydrochloride). After 20 minuts the solution was diluted to 4 ml with distilled water and absorbance was read at 540 nm on Toshniwal's VIS-Spectrophotometer. The amount of nitrite was estimated from a standard curve of sodium nitrite prepared in a similar manner.

(b) Nitrite reductase (E.C. 1.6.6.4) :

The activity of enzyme nitrite reductase (NiR) in root, stem and leaf was determined by the same method described already for nitrate reductase (NR) except that KNO_3 was replaced by 20 mM KNO_2 in the incubation medium and incubation was done in light. The KNO_2 present in the incubation medium was determined by reading the optical density of the reaction mixture containing 0.4 ml incubation medium, 0.3 ml of 1%sulfanilamide in 2 N HCl, 0.3 ml

of 0.20% NEEDA and 3 ml distilled water. The difference between the two readings, one at 0 min. and the other after the enzymatic reaction gives an amount of KNO_2 utilised (reduced) by the enzyme. Blank was prepared by mixing 0.4 ml of NiR incubation medium (without material), 0.3 ml sulfanilamide, 0.3 ml NEEDA and 3 ml distilled water. The amount of nitrite was estimated from a standard curve of sodium nitrite prepared in a similar manner.

(c) Effect of NaCl on enzyme activity :

In vitro effect of increasing concentrations of NaCl on the activity of enzymes NR and NiR was studied. Assay of the enzymes were done by the method of Jaworski (1971), already described in the text. The concentrations of NaCl tested for enzyme activity were 1 mM, 2mM, 3 mM, 4 mM and 5 mM The results are discussed separately.

2.2.7 Seasonal variations in physico-chemcal consttuents :

Entire plant of E. geniculata was analysed on fifteenth of each month throughout the year, for its pnysico-chemical constituents.

For estimatiion of physico-organic constituents fresh and oven dried material were used. While inorganic constituents were estimated from oven dried ($60^{o}C$) materials.

i) Physico-organic constituents :

pH of the leaf extract was determined by pH meter (NAINA, NPP. 300). While moisture and dry matter contents were determined by oven drying method as described by Patil (1980). Titratable acid number (TAN) was determined by the method of Thomas and Beevers (1949). The method described by

Arnon (1949) was followed for estimation of chlorophylls. Total nitrogen and proteins were estimated by the method of Hawk et al.,(1948). All these methods are described earlier in the text.

ii) Inorganic constituents :

To estimate inorganic constituents, plant samples (root, stem, leaves) were digested by wet digestion method of Toth et al., (1948). 0.5 g oven dried powdered sample was taken in a 100 ml. clean corning beaker and to it 15 ml. of concentrated Nitric acid was added. The beaker was covered with watch glass and was kept at room temperature till the initial reaction subsided. Then it was heated on a gas flame till all the particles of plant material were dissolved. The digest was then cooled to room temperature and to it 10 ml. of 60% Perchloric acid was added. It was then heated strongly until a clean and colourless solution resulted. Heating was stopped when the volume of extract was reduced to approximately 2-3 ml. It was then cooled and transferred quantitatively to a 100 ml volumetric flask. Finally volume was adjusted to 100 ml with distilled water. On next day the diges t was filtered through Whatman No.1 filter paper. The filtrate was used for the estimation of various inorganic elements except silicon (Si), where residue was used to estimate silicon.

Na^+ and K^+ were estimated with the help of Flame Photometer (Elico CL-22 A S.No. DO 390 Bombay). While Ca^{++}, Mg^{++}, Mn, P, Fe, Cu^{++}, Zn^{++}, Ni, Co, Pb, Cd were estimated with the help of Atomic Absiorption Spectrophotometer (Perkin Elmer No. 3030).

Silicon (Si) was estimated by the gravimetric method described by Shouichi et al., (1972). The residue obtained on the filter paper in the procedure

described earlier, was used for the estimation of silicon. This residue was first dried in oven, transferred to crucible and weighed accurately. It was then ahsed in furnace for one hour at about 400-500^{o}C. After cooling to room temp erature in desiccator the crucible with ash was weighed again. The difference in the two weights was taken as a measure to calculate the percentage of silicon in the plant material.

2.2.8 Pathophysiology :

Pathophysiology of E. geniculata infected with Melampsora is tried. Healthy and infected leaves were collected from the campus of Yashwantrao Chavan College of Science, Karad.

Fresh leaves of healthy and infected plants were used for enzyme assays and estimation of organic constituents. Oven dried leaves of healthy and infected plants were used for determination of total nitrogen, total proteins and inorganic constituents. The experimental methods are already described in this chapter.