CHAPTER II

Material and Methods

1) MATERIALS :

The seeds of following Crotalaria L. species under study were obtained from Regional Plant Introduction Station, Bestville U.S.A. : 1) C. falcata, 2) C. Lieoloba, 3) C. maxillaris, 4) C. ochroleuca and 5) C. stipularia.

2) METHODS :

I) Cytological Studies

a) Karyotypic Studies

i) Pretreatment :

The seeds of Crotalaria L. species were treated with sulphuric acid by continuous shaking for 5 – 10 minutes in order to over come dormancy and ensure germination of seeds. Then the seeds were throughly washed with water and were kept for germination on moist blotting paper in corning petriplates.

The somatic chromosome number was determined from the root tips. Actively growing root tips were excised and washed followed by treatment with aqueous saturated solution of paradichlorobenzene, for 3 – 4 hours in a freezer at 12 ^{o}C to accelerate the reaction of condensation and separation of chromosomes. After 3 –4 hours treatment the root tips were

washed and fixed into acetic alcohol (1:3). After 24 hours of fixation root tips were transferred to 70% alcohol and stored at 7 ^{o}C.

ii) Preparation of slide for microscopic observation :

The pretreated and fixed root rips were washed in 45% acetic acid and hydrolysed in 1 NHCl at 60 ^{o}C by gently heating over a spirit flame for 2 – 3 minutes after cooling the root tips were then washed thoroughly and then transferred to freshly prepared 2% aceto orcein. The meristematic portion of the root tip was carefully secured on the microslide and squashed in a drop of 2% aceto orcein by putting the coverglass. The slide was gently tapped with the blunt end of the needle. Excess of stain was squeezed out by pressing the slide with thumb holding on blotting paper. The slide was sealed with wax. The selaed slide was stored overnight to deepen the intensity of staining, so that it facilitated better study and photography for karyotypic studies. Microphotographs of well spread metaphases were taken from temporary and permanent preparation using MFAKS System of JENAVAL CZ microscope.

Slides were made permanent following butyl alcohol acetic acid series method. Seal of the coverglass was carefully removed with blade and slides were inverted on a glass rod facing coverslip downward in petridish containing tertiary butyl alcohol and acetic acid mixture in proportion of 1:2

for dehydration. After the coverslip fell off, the slide and coverslip was transferred further to a petridish containing a mixture of tertiary butyl alcohol and acetic acid in proportion of 1:1. In this petridish the slide was kept for 2 – 4 minutes and then lastly transferred to a petridish containing pure terriary butyl alcohol for 2 – 4 minutes for complete dyhydration. Slides and coverslips were mounted separately with DPX mounting medium.

For determining the length of chromosome, five plates were studied and average length of each chromosome was calculated from the data obtained. For karyotypic analysis the method of Levan et al., (1964) has been followed and idiogram of chromosomes was made. Karyotype symmetry has been analysed using Stebinns (1958) system of classification based on relative arm-length and arm-ratios. TF% was calculated as given by Huziwara (1962) while relative length (TCL%) and S% by applying following formulae :

$$\text{Relative length (TCL\%)} = \frac{\text{Chromosome length of longest chromosome}}{\text{Absolute length}} \times 100$$

$$\text{Relative length of shortest chromosome (S\%)} = \frac{\text{Length of shortest chromosome}}{\text{Length of longest chromosome}} \times 100$$

$$\text{Total form (TF\%)} = \frac{\text{Sum total of small arm length}}{\text{Total length of chromosome}} \times 100$$

b) Mieotic Studies

i) Fixation of buds

Young flowers buds of Crotalaria L. species under study were fixed in freshly prepared Carnoy's fluid in morning hours between 6.00 to 6.30 a.m. and after 24 hours of fixation, they were transferred to 70% alcohol and stored in a refrigerator.

ii) Preparation of slide for microscopic observations :

Anthers were dissected out in 45% acetic acid and approximate size of anther was carefully screened. Selected anthers were hydrolysed in 1 NHCl by gently heating over a spirit flame for few seconds followed by washing in water. The anthers were teased on the microslide in a deop of acetocarmine stain, which brought out the group of pollen mother cells from anther. A clean coverglass was placed on it, the slide was gently warmed on a spirit flame to achieve proper spreading. Excess of stain was squeezed out by pressing the slide on a clean blotting paper with thumb. The slide was sealed with wax for temporary observation.

Different stages of mieosis were studied from this preparations. The microphotographs were taken using MFAKS System of JENAVAL CZ microscope.

The slide was made permanent following butyl alcohol acetic acid series method as described earlier.

c) Pollen Fertility

Pollen fertility was determined on the basis of acetocarmine stainability technique. In this method matured anthers were freshly harvested. They were broke open by teasing the anthers with needle in a drop of 1% acetocarmine on a clean microslide. The pollen grains were allowed to spread uniformly and covered with coverglass. The slide was slightly warmed and excess stain was blotted out. The preparation was observed under microscope. The sterile pollen grains did not take stain. The pollen stained by 1% acetocarmine were considered to be fertile. One thousand pollen grains were analysed based on stainability, fertility percentage was determined.

II) Growth

The seeds of Crotalaria L. species under study were treated with sulphuric acid by continuous shaking for 5 - 10 minutes to break the dormancy. The seeds were then thoroughly washed with water and sown in pots and plots having fertile soil.

After completion of 20 days growth from planting day they were used for growth analysis. Growth analysis was done periodically after every 20 days interval from planting upto 80 days growth period.

Fifteen plants were carefully uprooted and washed thoroughly with water to remove dust particles on the surface of plant parts and blotted to dryness. This plant material was analysed for the growth parameters as, average height of a plant, average shoot length, average root length, number of leaves per plant, fresh weight^{-1} and dry weight^{-1}. From the above data, leaf area plant^{-1}, Relative growth rate (RGR), Net assimilation Rate (NAR) and Leaf Area Ratio (LAR) were calculated by applying following formulae :

$$1.\ RGR = \frac{\log_{10}(W_2 - W_1) \times 2.303}{t_2 - t_1}$$

$$2.\ NAR = \frac{2.303(W_2 - W_1)\ \log_{10}(A_2 - A_1)}{(A_2 - A_1)\ (t_2 - t_1)}$$

$$3.\ LAR = \frac{2.303\ (A_2 - A_1)\ \log_{10}(W_2 - W_1)}{2.303\ (W_2 - W_1)\ \log_{10}(A_2 - A_1)}$$

After harvesting 100 seed weight was determined.

III) Physiological Studies

When the plants were of 65 days old, were used for determination nitroten content, crude protein content, nitrogen content and activity of enzyme nitrate reductase.

1) Estimation of Nitrogen and Crude Proteins

Nitrogen was estimated by method of Nawk et al., (1948).

1 g of fresh plant material was digested in Kjeldahl flask containing 10 ml sulphuric acid (1:1) dilution. A pinch of micro salt and few glass bids were added. This was digested on low flame till a colourless solution was obtained at bottom flask and then it was cooled and then volume was adjusted to 100 ml with distilled water. Then it was filtered next day through Whatman filter paper No. 1 The filtrate was used for estimation of nitrogen.

1 ml of this filtrate was taken in Nesselors tube. In other tubes different concentrations of standard ammonium sulphate (0.05 mg.N/ml) were taken. One tube was kept as a blank without any ammonium sulphate. To these tubes were added a drop of 8% potassium bi sulphate and volume was made 35 ml with distilled water. 15 ml of Nesselors 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 of distilled water) in proportion of 4:5. Reaction mixture was throughly mixed and absorbance was recorded at 510 nm on UV-VIS-double beam spectrophotometer (Shimadzu 190) using reagent blank.

The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate. Nitrogen content is expressed as, g/100 plant material (fresh tissue).

Crude proteins were calculated by multiplying the total nitrogen value by factor 6.25.

ii) Nitrate Content

Nitrate content was estimated following the method of Jaworsky (1971). After determination of NR activity, the plant material from incubation medium was carefully removed and washed first with distilled water and then with 0.2 M phosphate buffer pH 7.5. It was then homogenised in 10 ml 0.2 M phosphate buffer pH 7.5. The extract was filtered through whatman filter paper No. 1. The filtrate was used for the estimation of nitrate (NO_3^-). Nitrate forms a blue coloured complex with diphenyl-amine H_2SO_4 reagent (Kolhoff and Nopone, 1933). 0.1 ml of extract was added to 1 ml distilled water and 1.8 ml diphenylamine H_2SO_4 reagent (1 g diphenylamine 100^{-1} ml H_2SO_4). The reaction mixture was vigorously shaken and kept for 10 minutes for colour development.

The absorbance was read at 590 nm on UV-VIS-double beam spectrophotometer (Shimadzu 190), distilled water was used as blank.

The standard curve was prepared for 1 m KNO_3 nitrate content was calculated and expressed as mg, gm^{-1} of fresh tissue.

iii) In vivo assay of Nitrate Reductase (E.C.1.6.6.1)

The method used in assaying in vivo nitrate reductase (NR) (E.C. 1.6.6.1) activity was of Jaworsky (1971).

The fresh plant material was thoroughly washed with distilled water and blotted to dryness. 0.5 g plant material was cut into pieces (leaves were cut into small disc's of about 0.5 cm^2) and incubated in 10 ml incubation medium containing 1 ml, 1M KNO_3; 2 ml, 5% n-propanol; 5 ml, 0.5% Triton-X-100, for 1 hour in dark. After 1 hour, 1 ml of reaction mixture was taken out for determination of nitrite and was mixed with 1 ml each of 1% sulfanilamide in 1 M HCl and 0.02% NEEDA (N-1-Naphthyl ethylene diamine dihydrochloride). The absorbance was read at 540 nm on UV-VIS-double beam spectrophotometer (Shimadzu 190) using reageant blank.

The standard curve was prepared with 0.03 mM KNO_3 (0.0026 mg NO_2 ml^{-1}) against a mixture of 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA as a blank. Enzyme activity is expressed as µg of NO_2 liberated g^{-1} fresh tissue h^{-1}.

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