MATERIALS AND METHODS

M A T E R I A L S A N D M E T H O D S.

MATERIALS :

Fresh seeds of diploid *Clitoria ternatea* L.(2n=16) variety blue solitary flower were collected from germ plasm collection maintained in the experimental Garden of P.G.Department of studies in Botany, Karnatak University Dharwad and used in the present investigation.

MUTAGENS :

Two chemical mutagens namely Ethyl Methane Sulfonate (EMS) a monofunctional alkylating agent with molecular weight 124.2 gms of Uibicheme limited Middlesex U.K. make and Maleic Hydrazide (MH), a white crystaline with molecular weight 119.12 gms obtained from Chem Linz A.G. Austria were used in the present study.

METHODS OF SEED TREATMENT :

To begin with, the pilot experiments were conducted to determine the suitable concentrations for the further studies. The suitable concentration was decided by 50 percent reduction in the germination percentage for each mutagen. The LD_{50} concentration for EMS was 0.25% and for MH 1.5 mM concentration for 24 hrs treatment.

PREPARATION OF SOLUTIONS:

The treatment solution of EMS prepared in glass-distilled water with 0.1 M phosphate buffer at pH 7 and MH solution prepared in glass-distilled water without any buffer. The pH of the MH solution was observed to be 4.2. Room temperature during treatments was 25 ± 2°C.

TREATMENTS:

Morphologically uniform sized and with 12.5 percent moisture content seeds were initially treated with concentrated sulphuric acid (H_2SO_4) for 15 minutes to break the dormancy. Treated seeds were washed thoroughly in running tap water for two hours and then soaked in distilled water for 12 hours. The presoaked seeds were placed in between two blotting papers for surface drying for an hour. Then these seeds were treated in three concentration of EMS (0.1,0.15 and 0.2%) and MH (0.01, 0.1 and 1.0 mM) for 6, 12 and 24 hours of durations with 1:5 volume of seeds and mutagenic solution with intermittent shaking. At the same time similar set of seeds sample were treated with distilled water as control. At the end of each treatment seeds were subjected for post treatment thorough washing for four hours in running tap water. For each treatment 450 seeds were treated. One hundred seeds of each treatment and control were grown in

germinating slots according to the method suggested by Myhill and Konzak (1967) to study the parameters such as germination percentage and seedling height. Fifty seeds were germinated on moist blotting paper in petriplates for cytological observatios and similar set was kept as control, whereas, remaining 300 seeds were sown in the field with three replications with a distance of 2 feet between rows and 1 feet between plants in a randamised block method along with control for M_1 observations. All the treated, control and mutants were grown under uniform conditions.

OBSERVATIONS IN M_1 GENERATION :

Effect of mutagen on germination, seedling growth (shoot length and root length), mitosis, meiosis and pollen fertility were studied.

GERMINATION :

Observation of germination was recorded at 8 hours interval after treatment till 15th day of sowing. Emergence of radical was considered as criterion for germination.

SEEDLING GROWTH :

The seedling growth was determined based on the root and shoot lengths on 15th day after sowing. The shoot length was measured from the base of stem (transition zone) to the tip of the apical bud and root length from the point of

emergence of radical to the tip of the root in cetimeters for the main root.

CYTOLOGICAL STUDIES :

Mitosis

Fifty seeds of each treatment and control were germinated in petriplates with moist filter paper at room temperature (25±2°C). Root tips of various treatment and control were fixed when the root tips were of 1.5 cm length in acetic alcohol (1:3) for 8 hours. They were later on transferred to 70% ethanol and stored in refrigerator. Squash preparations were made with the help of leucobasic fuchsin stain (Darlington and La Cour, 1947). Semipermanent slides were made by using 1% aceto carmine stain. At least 30 slides were studied for each treatment and mitotic chromosomal aberrations were scored. Similar number of slides were also examined for control treatment. Photomicrographs were taken from semipermanent slides using C.Z microscope under apochromatic objective.

Meiosis :

Meiotic observations were made by fixing suitable flower buds from 25 randomly selected plants for each treatment and as well as for control. Flower buds were fixed in Carnoy's fluid B and stained in 1% acetocarmine solution.

Chromosomal abnormalities were scored by analyzing different meiotic stages. Slides were made permanent with acetic acid and butanol shedule and mounted in euparol (Celarias, 1956). Photomicrographs were taken from the permanent slides using C.Z. microscope under apochromatic objective.

POLLEN STUDY :

Twenty five flowers were randomly selected from each treatment and also from control plants to study the pollen fertility. Pollen grains from just dehised anthers from flower were smeared in a mixture of 1% acetocarmine and 50% glycerin (1:1) and examined under microscope. Complete and darkly stained pollen grains were considered as fertile, while partially, unstained, and shrivelled pollen grains considered as sterile. In each treatment and contol at least 10,000 pollen grains were studied to know average pollen fertility percent.

SURVIVILITY :

Survived plants were calculated at maturity with number of seeds germinated per concentration and as well as in the control.

CHLORO AND MORPHO VARIENTS :

Chlorophyll and morphological varients were recorded throughout the vegetative and reproductive phases of the

plants of M_1 generation. Wherever any interesting varients were noticed, they were tried to maintain through sexual or vegetative propagation.

QUANTITATIVE CHARACTERS :

Twentyfive randomly selected plants were tagged for recording the following observations viz. plant height, number of node to first flower, number of branchs/plant, number of pods/plant, number of seeds/pod were studied on the plants grown in the field.

SEED PRODUCTION :

Twenty five plants were selected randomly in each treatment and five pods per plant were selected to study the seed production by counting total number of potential sinks and number of normal seeds formed. Observation was also carried out for control.

OBSERVATION IN M_2 AND M_3 GENERATIONS.

Seeds of 25 normal looking plants of M_1 generation were selected at random and were collected on individual plant basis from each treatment and control. They were used to raise the M_2 generation on a plant to a row basis.

The following observations were recorded in the M_2 and M_3 generations. Cytological observation, frequency and spec

trum of chlorophyll, viable mutations, calculation of mutagenic effectiveness, efficiency values based on chlorophyll and viable mutation frequency and critical assessment of induced variations in different quantitative characters were made.

CHLOROPHYLL MUTATIONS :

The chlorophyll mutations were scored in the field when the seedlings were of 15 days old. Chlorophyll mutations were classified according to method suggested by Gustafsson (1940). The frequency of chlorophyll mutants were calculated according to Gaul (1960) i.e. number of mutants/ 100 M_2 plants.

MUTAGENIC EFFECTIVENESS AND EFFICIENCY :

The mutagenic effectiveness is a measure of the frequency of mutations induced by a unit dose of mutagen (KR or time X concentrations) while mutagenic efficiency gives an idea of the proportion of mutations in relation to biological damage such as lethality, seedling injury, chromosomal abnormalities and pollen sterility. Mutagenic effectiveness and efficiency of different mutagens were calculated according to the formulae suggested by Konzak *et al.*(1965).

$$\text{Mutagenic effectiveness} = \frac{\text{Mutation frequency (M/F)}}{\text{dose or (time X concentration)}}$$

$$\text{Mutation efficiency} = \frac{\text{Mutation frequency (M/F)}}{\text{Biological damage}} \times 100$$

= MF/L, MF/I, MF/MI, MF/ ME and MF/PS

Where MF = Mutation frequency of M_2 and M_3 generation

L = Percent lethality in M_1 generation

I = Percent seedling injury in M_1 generation

MI = Percent Mitotic abnormalities in M_1 generation

ME = Percent Meiotic aberrations in M_1 generation

PS = Percent pollen sterlity in M_1 generation.

MUTATION RATE :

The mutation rate was calculated by following formula

$$\text{Mutation rate (MR)} = \frac{\text{Sum of values of effectiveness or efficiency of a particular mutagen}}{\text{Number of treatment of that particular mutagen.}}$$

This gives an idea of mutations induced by a particular mutagen irrespective of concentration and duration of treatment.

QUANTITATIVE CHARACTERS :

From each treatment 50 plants were randomly selected

to record the data on various quantitative characters in M_2 and M_3 generations. Similarly, 50 plants were also selected randomly from the control for comparative study. Data on following quantitative characters have been recorded.

Plant height :

The height of plant was measured on 60th day of sowing from the bottom to tip of the plant.

Number of nodes at first flowering stage :

Number of nodes from the base of plant to first flowering stage on the main stem were counted and considered as total number of nodes up to first flowering stage.

Number of branches :

All branches of plant were taken into account for total number of branches per plant.

Rachis length :

It was measured in centimeters from the base of the rachis to base of terminal leaflet at first flowering stage.

Length and Breadth of leaflet :

Length was measured from the base to tip of the leaflet and breadth was measured in middle of the leaflet, for

three terminal leaflets.

Number of leaves :

All leaves on main stem as well as branches were counted as total number of leaves per plant.

Number of days to 50 % flowering :

Number of days taken for the opening of the first flower from the date of sowing till 50% flowering in the population in each treatment were recorded for treated as well as for control population also.

Days to flower :

Minimum and maximum number of days taken for the opening of the first flower were recorded in each treatment and control.

Number of flowers per plant :

Number of flowers on entire plant were considered to calculate the number of flowers per plant.

MACROMUTATIONS (viable mutations) :

Mutation that can be scored externally and which affect the morphological characters of the plant were considered as macromutations. They were scored during throughout the life cycle of the plants in M_2 and M_3 generations. All

such plants were harvested separately in M_2 generation. Their true breeding nature has been ascertained during subsequent generations.

A range of flower colour mutations could be observed in M_1 and M_2 generations and their true breeding behavior was confirmed in subsequent generations. For the identification of leaves, flowers and seed coat colours and their grades, the Wilson colour chart volumes (1938, 1941) were referred.

MICROMUTATIONS :

These are the mutations which cannot be detected visually but need biometrical analysis for their detection.Such plants were subjected to biometrical analysis and screened accordingly.

STATISTICAL ANALYSIS :

Various statistical data were computed using the following formulae.

$$\text{Mean} = \frac{\Sigma X}{n}$$

$$\text{S.D.} = \sqrt{\text{variance}}$$

$$\text{S E} = \frac{SD}{\sqrt{n}}$$

$$\text{C V} = \frac{\text{S D X 100}}{\text{Mean}}$$

$$C\ D = S\ E\ m \pm \sqrt{2} \times t.e.d.f$$

(error degree of freedom)

$$t = \frac{\bar{X}-\bar{Y}}{SEd}$$

Where X = Sum of all the observations

n = Total number of observations

S D = Standard Deviation

S E = Standard Error

C V = Coefficient of variation

C D = Critical Difference

S E m = Standard Error of mean

$\bar{X} - \bar{Y}$ = Observed difference between the grand mean

S E d = Standard Error of difference

Test of significance:

Test of significance of difference between means of control and other treatments were made with the help of critical difference (CD), 't' test was used for testing the significance difference between the grand mean of the mutagens.

The conclusion were drawn considering the critical difference (CD) and 't' values at 1 and 5% level. The ANOVA was calculated as per Daniel (1977) following abbreviations

were used.

S V = Source of variation

D F = Degree of Freedom

S S = Sum of squares

M S S = Mean sum of squares

F = Test value

Treatment = Concentration of mutagen

Block = Duration of treatment

BIOCHEMICAL ANALYSIS :

Crude leaf protein content in the different mutants of *C.ternatea* and in control was estimated by Micro Kjeldhl method and protein percentage was estimated by multiplying the nitrogen with 6.25 factor (AOAC, 1975).

MORPHOLOGICAL CHARACTERS OF *C.ternatea* L.

It is a perennial climbing herb with 34.50 cm in height and with 5 trailing branches on 60th day. Number of node on the main stem to first flowering stage are 10. Stem is sparsely pubescent and terete. It takes 150 to 160 days to complete one life cycle i.e from germination to maturation of seeds. Leaves are 27 in number, imparipinnate, leaflets 5 to 7. Size of lateral leaflets are 3.39 X 2.77 cm and terminal leaflet 3.58 X 2.84 cm with short pulvinate petiole. Eliptically oblong, apex obtuse, margin entire,

glabrous above, pubescent beneath. Length of rachis is 7.30 cm, stipulate , filiform. It takes 64 days to flower. The peak of flowering period is between the month of August to September and number of flowers are 24. Flowers solitary, zygomorphic, bisexual, axillary with two large, roundish obtuse persisting bractioles. Epicalyx two, orbicular, cordate, pubescent, much enlarged infront. Calyx tube green in colour with one large tooth, veined, hairy and persistent. Corolla Papilionaceous, standard, dark blue-purple with light blue striations and orange-yellow colour at the centre. Stamen 10 diadelphous (9+1). Ovary superior, flat, monocarpellary, uniloculary, multiovulate and highly self fertilized. Pods flattened 10 cm in length, 1.0 cm in breadth, linear, sharply beaked, sparsely appressedly hairy, valves twisting after dehiscence. Tender pods are green in colour when it matured turn to yellowish-brown in colour. It takes 60-70 days for maturation for pods. Number of pods are 21, seeds 8 per pod with brownish green in colour . Seeds are dormant because of hard seed coat.