MATERIAL AND METHODS

The selfed seeds (with 13% moisture content) of forage legume Desmodium tortuosum (SW.) DC have been used as an experimental material. The seed material initially was obtained from Fodder division of University of Agricultural Sciences, Dharwad and later it was grown in the Experimental Garden of Post Graduate Department of Botany, Karnatak University, Dharwad for two years.

Healthy and morphologically uniform sized seeds (total of 350 seeds per concentration) have been treated with chemical mutagens diethyl sulphate (dES) and ethylmethane sulphonate (EMS). Out of 350 seeds, 50 seeds were used for cytological analysis, 100 seeds for seedling parameters and remaining seeds of each treatment were used for further study.

Administration of mutagenic treatment

The treatment conditions for the present study have been tabulated in the experimental protocol (Table 1).

Pretreatment:

The pretreatment soaking was carried out in double distilled water for four hours at the room temperature, 25 $\pm$ 1^{o}C. Presoaked seeds have been used in the present experiment since presoaking increases the cell membrane permiability and helps in the uptake of mutagenic solution (Mikaelsen et al., 1968 ; Kamra and Brunner, 1977)

TABLE -1 : EXPERIMENTAL PROTOCOL

Sl No	Mutagenic Chemicals	Abbr.	Make	Concentration in %	Pretreatment soaking	Treatment duration	Temp	pH	Buffer	Post treatment washing
1.	Diethyl-sulphate.	dES	E.Merck Germany	0.10, 0.15, 0.20 &0.25	4 hrs.	8, 16 & 24 hrs.	16°C	7	Phosphate	2 hrs.
2.	Ethylmethane-sulphonate	EMS	Ubi.Chem, Stains, Middlsex U.K.	0.20, 0.25, 0.30 &0.35	4 hrs.	8, 16 & 24 hrs.	25±1°C	7	Phosphate	2 hrs.

Concentrations:

The concentrations of both the mutagen have been determined on the basis of LD_{50} at germination level. The concentrations selected were 0.10, 0.15, 0.20, 0.25% for dES and 0.20, 0.25, 0.30, 0.35% for EMS. The range of the dES and EMS concentrations recommended for the mutagenic treatment is 0.1 to 0.6% and 0.3 to 1.5%, respectively (Mikaelsen et al., 1968 ; Savin et al., 1968) Although, this range is advocated for cereal crop plants but, also proved to be suitable for the other plants.

The presoaked seeds were air dried at room temperature for one hour and then treated with said concentrations of dES and EMS for different treatment durations (Table 1).

Volume of the mutagenic solution:

The volume of the mutagenic solution per seed was maintained as 2 ml/1 seed. Similar proportion of volume was maintained for control. This proportion was administered to facilitate the uniform absorption of mutagenic chemical by individual seeds. The treatment solution was gently shaken intermittantly during treatment period.

Treatment duration:

In the present study, the mutagenic treatment have been conducted for 8, 16 and 24 hrs. durations. The three treatment durations have been selected following the report that, the high mutagenic

effectiveness is obtainable when embryos are treated in metabolic stages in relation to DNA synthesis (Natarajan and Shivasankar 1965; Savin et al., 1968; Gopal-Ayengar et al., 1969 ; Khalatkar et al., 1977).

Buffer:

Unbuffered mutagenic solution produces acidic products thus reducing the mutagenic potentiality of alkylating agents (Ramanna and Natarajan, 1965). To check such events, the 0.1 M phosphate buffer with pH 7 was used to prepare to mutagenic solutions. The use of phosphate buffer with pH range of 6 to 11 have been suggested for the mutagenic treatments of dES and EMS (Kamra and Brunner, 1977).

Temperature:

The temperature of the treatment conditions have been determined accounting the half life period of dES and EMS. Since, the half life period of dES is very short at room temperature (25 $\pm$ 1^{o}C), the treatment was conducted at lower temperature (16^{o}C). The solution was renewed every three hours during the treatment period (The half life of dES of 20^{o}C is 3.57 hrs , Kamra and Brunner, 1977). The EMS treatment was administrated at room temperature (25 $\pm$ 1^{o}C) The half life of EMS at 30^{o}C is 25.9 hrs (Kamra and Brunner, 1977).

Post treatment:

The treated seeds were subjected to the post treatment washing

thoroughly for two hours under running tap water. The post treatment was carried out to remove the traces of mutagenic chemicals from the seed coat which otherwise hinder the germination process.

Management of M_1, M_2 and M_3 generations:

The treated seeds along with control were transferred to the experimental garden initially to the pot for two weeks to study the M_1 generation. Later seedlings were transferred to the field following randomized block design along with control. Seedlings were sown at a distance of 30 cms apart and 90 cms between the rows. The seedlings were grown under uniform conditions. At maturity, the pods were collected concentration and treatment duration wise from fifty normal looking plants of M_1 generation following plant progeny bulk method (Konzak and Mikaelsen 1977). The M_2 generation was subjected for the detection of chlorophyll and morphological mutations. The plants of interest were isolated and tagged with the label. The studies on the quantitative traits have been made in M_2 generation onwards. In M_2, the seeds were collected from individual plant to raise the M_3 generation in a row to study the inheritance pattern of quantitative traits. Similarly the plants of interest, the seeds were collected on individual plant basis and grown on plant to row system to confirm mutated characteristics encountered in M_2.

Parameters studied in M_1 generation:

Following parameters have been studied in M_1 generation to know the mutagenic sensitivity of dES and EMS.

Seed germination:

The total of hundred treated seeds per concentrations were placed in sterilized petriplates containing moistened blotting papers. The care was taken to keep blotting papers moistened always by sprinkling the water at morning hours. The seeds which developed radicles and plumuleswere considered as a germinated seeds. The percent germination was estimated per 100 seeds on 7th day.

Seedling height:

The seedling height measurement was taken on 15th day using PVC growing racks following the method of Myhill and Konzak (1967)

Plant height:

The plant height was measured at maturity. This measurement was scaled in centimeters from the region of the initiation of roots to the tip of the plant.

Number of the flowers per inflorescence:

The inflorescence axis of main branch have been considered to record the total number of flowers per inflorescence. The mean flower number of twenty five observations per concentration have been tabulated.

Plant survivility:

The number of the plants surviving at maturity were counted per concentration. The per cent survivility was estimated per number of the seeds treated per concentrations.

Cytological observations:

a) Mitosis:

The quantum of somatic chromosomal aberrations resulted by the treatment of mutagenic chemicals (dES and EMS) have been determined by analysing the mitosis in root tip of treated seeds as well of control seeds. Total of fifty seeds per concentrations were placed seperately in sterilized petriplates with moistened blotting paper. The root tips of half centimeter length only were fixed for 24 hrs in Cornoys fixative I (Acetic acid + Alcohol, 1:3) at room temperature 25 $\pm$ 1°C. The root tips were taken between 9.45 to 10.30 am since, during this period the mitotic activity was maximum in control. After 24 hrs of fixation, the root tips were transferred to 70% alcohol for further preservation.

For mitotic preparation, the root tips were hydrolised in 2 drops of 1 N HCl and 8 drops of 1.5% aceto-orcine and squashed in 1% aceto-orcine. The slides were made permanent adopting acetic acid butanol series (Celarier, 1956). The mitotic metaphase and anaphase have been screened for the detection of chromosomal aberrations. The microphotographs were taken in Carl Zeiss microscope under 10 x 100 apochromatic objective.

b) Meiosis:

For meiotic analysis, the flower buds of suitable size were collected from randomly selected plants of concentration and treatment duration

wise during 8.30 to 9.30 am. The flower buds along with control buds were fixed in Cornoys fixative II (Acetic acid + Alcohol + Chloroform, 1:6:3), After 24 hrs of fixation, the flower buds were transferred to 70% alcohol for further preservation.

The anther smear preparations were made in 1.5% aceto-carmine stain with the trace of ferric acetate (Belling, 1928). The permanent slide preparation was made in acetic acid-butanol series (Celarier 1956). Dividing PMC's were screened treatment wise and also in control. The meiotic anomallies were observed in metaphase I and anaphase/telophase I and II. The microphotographs were taken under 10 x 100 oil appochromatic objective of Carl Zeiss microscope. The meiotic study was computed for M_1 , M_2 and M_3 to know the effect of mutagenes on meiotic system in these generations along with control.

Sterility:

a) Pollen sterility:

The pollen count per concentration have been made to estimate the degree of pollen sterility in treated material as well as in the control. These observations were made for M_1, M_2 and M_3 generations. Pollen grains were taken from ten randomally selected plants of each treatment. Observations were made on the basis of stainability of pollen grains in (1% Aceto-carmine and 50% Glycerine mixture (1:1). Shrunken, empty and unstained pollen graims were considered as a sterile one.

b) Number of seeds per pod:

The number of seeds per pod were noted to correlate the induced sterility with seed setting and yield. The first ten pods per main inflorescence per plant have been considered and the average value per concentration have been tabulated along with control. These observations were made for M_1, M_2 and M_3 generations.

Chlorophyll and Morphological variations:

The variations with respect to chlorophyll and morphological characters have been recorded in M_1 generation. The percentage of varients have been estimated on the basis of the plants surving at maturity.

Observations in M_2 and M_3

Chlorophyll Mutations:

The chlorophyll mutations were detected right from early seedling stage to the maturity in M_2 and M_3 generations and per cent occurrence have been estimated on the basis of number of the plants surviving at maturity. Chlorophyll mutations have been classified following Gustafsson (1940).

Macromutations:

The morphologically visible mutations have been screened in M_2 and M_3 generations. The plants with altered characteristics were

subjected for inheritance pattern. Following characters were studied, habit, branching pattern, number and size of the leaves, fertility characters, crude protein content and electrophoretic banding pattern. The per cent occurrence of morphological variations have been estimated on the basis of number of the plants surviving at the maturity. To facilitate photography, the mutant plants were transferred to the earthern pots of suitable size.

Mutagenic effectiveness, efficiency and Mutation rate:

The mutagenic effectiveness and efficiency have been estimated following the formulae suggested by Konzak et al. (1965).

$$\text{Mutagenic effectiveness} = \frac{MF}{xC}, \frac{MF}{tC}$$

$$\text{Mutagenic efficiency} = \frac{MF}{I}, \frac{MF}{L}, \frac{MF}{SP}, \frac{MF}{PS}, \frac{MF}{MI} \text{ and } \frac{MF}{ME}$$

Here MF = Chlorophyll mutation frequency

x = Number of the times reneved the solution (applied for dES)

t = treatment hours (applied for EMS)

C = concentrations of mutagenic solution.

I = Seedling injury in M_1

L = Lethality* in M_1

SP = Percent reduction in seeds per pods* in M_1

PS = Pollen sterility* in M_1

MI = Mitotic aberrations* in M_1

ME = Meiotic aberrations* in M_1

(* Percent of control)

Mutation rate:

$$\frac{\text{Sum of the mutagenic efficiency for perticular parameters}}{\text{Total number of treatments of perticular mutagen}}$$

Quantitative characters studied in M_2 and M_3 generation:

Randomly selected ten plants per row per concentration were considered to study the quantative characters in M_2 and M_3 generations along with control. The mean performance per concentration have been expressed with the help of statistical analysis. The studies on quantative characters have been confined for the following parameters.

1) Plant height:

The height of the plant was measured at first flowering and mean performance have been expressed in centimeter. The height was scaled from the bottom (the region of root initiation) to the tip of the plant.

2) Number of branches per plant:

The total number of branches (including secondary and tertiary) have been recorded per plant. The results have been expressed as mean performance per concentration.

3) Leaf size:

The length and the breadth of the basal leaf (one terminal and two lateral) of main branch have been measured in centimeter at first

flowering stage on main branch. The results have been expressed in term of mean leaf size per concentration. Two lateral leaflets of the leaf in D. tortuosum exhibited similar size thus, the size of only one lateral leaf let has been computed.

4) Number of the leaves per plant:

The total number of the leaves per plant have been estimated at the initiation of first flowering and the mean performance per concentration have been tabulated.

5) Number of the flowers per inflorescence:

The main inflorescence axis per plant have been considered to estimate the total numbers of the flowers per inflorescence per plant. The pooled data of ten observations per row per concentration have been expressed.

6) Number of pods per inflorescence:

The main inflorescence per plant have been considered to estimate the total number of the pods produced per inflorescence. The mean value of ten observations per row per concentration have been tabulated.

7) Number of the seeds per pod:

The first ten pods of main inflorescence per plant have been considered to estimate the number of seeds per pod. The mean value per concentration have been tabulated.

Statistical analysis:

The phenotypically invisible variability in quantitative characters induced by dES and EMS have been analysed statistically. Following statistical expressions have been used in the present study. The ANOVA have been employed following Daniel (1977).

Mean, $\bar{X} = \frac{\Sigma X}{n}$

Standard Deviation, SD $= \sqrt{\frac{\Sigma X^2}{n} - \bar{X}^2}$

Standard error, SE $= \frac{SD}{\sqrt{n}}$

ΣX = Sum of observations

n = Total number of observations.

T^2 = Square of grand total

Correlation factor, CF $= \frac{T^2}{kn}$

kn = Number of observations for treatment and Block.

Total sum of squares, SST = Sum of all squared observations - CF

Sum of squares for Blocks, SSBL $= \frac{\text{Sum of squared Block total}}{\text{Number of Treatment observations (k)}}$

Sum of squares for Treatments, SSTR $= \frac{\text{Sum of squared Treatment total}}{\text{Number of Block observations (n)}}$

Error sum of squares, SSE = SST - SSBL - SSTR.

ANOVA - Table for Treatments* and Blocks**

Source	SS (Sum of squares)	df (degree of freedoms)	MS (Mean square)	VR (Variance Ratio)
Treatments	SSTR	$k-1$	$\frac{SSTR}{k-1} = MSTR$	$\frac{MSTR}{MSE}$
Blocks	SSBL	$n-1$	$\frac{SSBL}{n-1} = MSBL$	$\frac{MSBL}{MSE}$
Error	SSE	$(k-1)(n-1)$	$\frac{SSE}{(k-1)(n-1)} = MSE$	

*Concentrations.

** Treatment durations.

Coefficient of variation $CV = \frac{SD}{\bar{X}} \times 100$

Critical Difference $CD = SEm \times \sqrt{2} \times t$

SEm = SE of mean

t = Table t value at error degree of freedom

't' test have been calculated as:

$$\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

$\bar{X}_1$ and SE_1 = mean and standard error of A.

$\bar{X}_2$ and SE_2 = mean and standard error of B.

(A represent always maximum mean compared to B).

Protein estimation:

The crude protein content of leaf has been estimated in control and mutant plants following classical- Kjeldahl method (AOAC, 1965).

The matured leaves of control and mutant plants have been collected at first flowering and oven dried at 60°C. The dried leaves were finally powdered using mortal and pestle. The leaf powder was stored in air tight glass bottles.

One gram of leaf powder (per sample) was digested in 20 ml of concentrated H_2SO_4 for 8 hours in presence of the pinch of catalyst (Mixture of $CuSO_4$ + K_2SO_4 , 1:2). Acid digestion was done in digestion chamber. Thus, the nitrogenous compounds were converted in to ammonium sulphate. The ammonium content was liberated by steam distillation in Kjeldahl aparatus. The content then titrated against 0.02 N HCl till the colour changes from bluish green to pink and the volume of HCl required for titration was considered to calculate nitrogen content in the distillate.

The resulted value of nitrogen content was multiplied by factor of 6.25 to obtain the crude protein content in the mutant samples and control. The crude protein content was expressed in percent. Protein estimation experiment was repeated thrice in order to verify the reproducibility of protein content.

Protein banding:

The morphomutants along with the control were subjected to study

the seed protein banding pattern by SDS-PAGE method (Laemmli,1970) in 10% gel. The Klamaphor electrophoretic unit was used in the present study.

Healthy, matured and fully dried seeds of each mutant sample and control were selected and powdered in mortal and pestle. One gram of seed meal was mixed in 0.10 ml phosphate buffer (pH, 7) on magnetic stirrer for 1 hour. The resultant slurry was squeezed through the cheese cloth and centrifused for 30 minutes at 3000 RPM. The suspension was decanted, filtered and clear supernatant was collected. To remove the phenolic compounds in the extract if any, 1 ml solution of 1% polyvinyl-pyrrolidone (PVP) was added to the tubes and stored as such in refrigirator for over night. The suspension was subjected to slow speed centrifugation (1000 RPM) again for 10 minutes. The solution was filtered again and resultant supernatant was stored at $0^{o}C$ in refrigirator.

The protein samples were mixed with 100 µl of the sampling buffer containing 60 mM Tris HCl, pH 6.8; 2% SDS; 10% Sucrose and 2% of 2- mercaptoethanol and boiled for 5 minutes at $100^{o}C$. The samples containing 150 to 200 µg of proteins were loaded in sampling wells of the slab by using hypodermic syringe. The tracking dye was added in each sampling well to mark the moving protein front. The anode and cathode chambers of the electrophoretic unit were filled with electrode buffer till it touches the gel.

The electrophoretic power set was connected to the unit and the power supply of 15 mA was allowed to run till the marker dye entered the

seperating gel. Later, the current of 48 mA was allowed to run till the marker dye reached to the bottom of the gel. The unit was kept in cold chamber during electrophoresis to avoid adverse effect of high temperature during experiment. The slab gel then removed gently and washed in water. The gel was then fixed in for 6 hrs in fixative (Acetic acid/Methanol/Water, 10:40:50, v/v/v). The slab gel was stained with silver nitrate stain. Reproducibility of protein band pattern was verified by three repetations of experiment. Finally, gel was photographed.

The diagramatic representation of slab gel was to made on the basis of visual observations The diagram was scaled (13 cm) to indicate the movement of protein fronts. Each protein band was numbered according to the relative position of origin. On the basis of staining intensity, the protein bands were graded as dense, medium, weak and very weak bands (Hirano, 1975). The Rm values were calculated considering the relative mobility to particular band on electrophoretic gel (Landizivisky and Hymowitz, 1979).

The Rm values for each band of mutant sample and control have been calculated as follows.

$$Rm = \frac{\text{Distance travelled by protein band}}{\text{Distance travelled by tracking dye}}$$