C. MATERIAL AND METHODS

Seeds of *Nigella sativa* L., obtained from local nurseries have been used in the present investigation.

*Studies on control samples*

Control seeds of *Nigella sativa* were allowed to germinate in petriplates lined with moist filter paper and in the experimental field plots for the following cytological studies.

*Karyotype analysis*

Karyological studies were performed by somatic metaphase and pachytene.

*Somatic karyotype*: Young healthy root tips were pretreated with 0.05% aqueous solution of colchicine for 3 hours at 22°C ± 2°C; this was followed by overnight fixation in 1:3 acetic alcohol and finally stained in 2% orcein-HCl mixture (9:1) for at least 2 hours. Root tips were squashed in 45% acetic acid.

Karyomorphology of the metaphase chromosomes was analysed considering the following parameters: (i) mean length
of individual chromosome measured in μ, (ii) relative length of each chromosome represented as percent length of longest chromosome in a species, (iii) r-index value for determining the nature of primary constriction in the chromosomes (Levan et al. 1964), (iv) form percent (P%) for each chromosome representing the short arm length as percent total chromosome length (Hirahara and Tatsuno 1967), (v) total form percent (TP%) represented the total length of short arms as percent total length of chromosome complement (Huziwara 1962), (vi) percentage of total chromatin length (TCL) has been determined from total length of a chromosome/totall length of chromosome complement X 100, (vii) disparity index or DI (length of the longest chromosomes - length of the shortest chromosome X 100), (viii) value of S% represents relative length of the smallest chromosome/relative length of the largest chromosome X 100, (ix) total haploid chromatin length measured in μ, and (x) presence or absence of secondary constrictions.

Idiogram was constructed in this case comparing randomly taken ten camera lucida drawings at a magnification of X 1750. Idiogram of a scattered metaphase plate has also been presented for convenience of study.
Pachytene karyotype

For the study of the pachytene chromosomes, flower buds of suitable size were fixed in acetic alcohol. Traces of ferric chloride were added to the fixative to intensify the staining of the chromosomes. The materials were kept in the fixative for overnight at low temperature (16°C) and then preserved in 70% ethyl alcohol for further studies.

Fixed anthers were smeared in one or two drops of 1% propiono-carmine. Slight warming and gentle tapping promoted excellent spreading and scattering of the bivalents. Analysis of the chromosome complement at pachytene was done in seven best P'Cs after screening a large number of them. Drawing of pachytene bivalents were made from temporary slides with the aid of a camera lucida using X 100 oil immersion objective and X 16 eye piece of Meopta microscope. The bivalents, were measured with the aid of a fine divider and the measurements were made in microns (\(\mu\)).

Individual bivalents at pachytene were identified by their absolute lengths, relative lengths, F% and arm ratios. The length of the satellite was included in the respective arm for calculating the arm ratios. After proper identification, the chromosomes were numbered from 1-6 in the descending order of their lengths.
In vitro studies

For this study, callus cultures were initiated from seeds.

The seeds of *Nigella sativa* L. were germinated following surface sterilization with 0.1% mercuric chloride for 10 minutes under sterile conditions. Callus cultures were initiated from hypocotyl segments, placed on agar (0.8%) slant medium in test tubes, each containing 10 ml of the medium. Murashige and Skoog's (1962) medium being supplemented with 2, 4-D (2 mg/l) and kinetin (1 mg/l) was used in the present experiment. The medium was adjusted at pH 5.8. After sufficient growth of the callus tissue (4 weeks) they were transferred to a fresh medium. Each subculture was made in a fresh medium at an interval of 30 days and maintained in a light-dark (16 hours - 8 hours) photoperiod at 22°C ± 2°C.

Cytological preparations were made from the callus tissues fixed in acetic-alcohol (1:3) during subsequent subcultures and stained following orcein-HCl procedure. Chromosomal variations were studied from the callus cells of different sub-cultures and number of telocentrics was scored accordingly.
Giemsa C-banding in somatic cells

The root tips grown in moist petri dishes were cut out to a suitable mean length of 2-3 cm and pretreated in 0.05% aqueous colchicine for 3 hours prior to fixation in acetic-alcohol (1:3) at 0°C-4°C for 1-3 days. The root tips were then hydrolyzed in 0.1N HCl at 60°C for 5 minutes, macerated and finally squashed in 45% acetic acid. The coverslips were removed in absolute alcohol, air dried and kept for further use. C-bands were obtained using the procedure outlined by Fiskejo (1974) with slight modifications.

The air dried slides were placed in 0.2 M HCl (denaturation for 1 hour at room temperature, which being rinsed in demineralized water, were placed in 2 X SSC for 1 hour 55°C-65°C (renaturation), and were again rinsed in demineralized water and finally stained with 5% Giemsa (Merck) solution diluted (1:20) in 0.2 M phosphate buffer (pH 6.8). The preparations were then rinsed in demineralized water, air dried and mounted in euparol.

Determination of mitotic cell cycle

For determination of mitotic cell cycle duration, the colchicine method of Van't Hof et al. (1960) and Van't Hof
and Sparrow (1963) has been followed. The technique involves production of a small population of tetraploid cells by the cytological action of colchicine in meristems composed of diploid cells. The time lapse between colchicine treatment and the appearance of tetraploid cells in the subsequent mitosis is equivalent to one mitotic cells cycle.

For this study, the roots were immersed in 0.35% colchicine for 1 hour and after washing they were allowed to grow in moist petriplates. The treated root tips were fixed in acetic alcohol (1:3) after 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours of treatment with colchicine, and this was followed by hydrolysis in 3N HCl for 10 minutes at 60°C. Then the root tips were stained as usual in 2% aceto-orcein and finally squashed in 45% acetic acid solution. Frequency of colchiploidy as well as normal diploid cells were scored after the duration of each treatment.

**Studies on induced mutation**

Seeds of *Nigella sativa* L. were treated with mutagens.
Mutagens used

Two mutagenic agents namely, X-rays and ethyl methane sulphonate \((\text{CH}_3\text{SO}_3\text{C}_2\text{H}_5)\) were used.

Method of treatments

X-ray : For each of the treatments with X-rays, 200 dry seeds (1.5% moisture content), suitably packed in single layer in small sealed polythene bags were taken. Facilities of X-ray irradiations were obtained from Jute Agricultural Research Institute, Barrackpore, West Bengal, in the year 1979. Seeds were irradiated with 4 Kr, 6 Kr, 8 Kr, 10 Kr, 20 Kr and 30 Kr of X-rays from a Philips X-ray machine using no filter. Time taken for 4 Kr irradiation has been 2 minutes 20 seconds and the distance of the material from the source was 10 cm.

Ethyl methane sulphonate (EMS) : Seeds presoaked in deionized water (12 hours) were pretreated with freshly prepared 0.2%, 0.75% and 1.0% solution of EMS (Kodak Chemicals, USA) dissolved in 0.2 M phosphate buffer. Treatments were performed for 2 and 4 hours with intermittent shaking at 27°C ± 2°C at a pH of 6.8. For each of the treatments with EMS 200 seeds were used.
Post mutagenic treatment

Irradiated seeds were sown in the well prepared field plots immediately after the treatments, while EMS treated seeds were carefully washed in running water before sowing in the field.

Field trials

Treated and control seed samples were grown individually in well prepared field plots and were sown in lines keeping a distance of 15 cm between the plants and 25 cm in between the lines. Arrangements were made for regular field operation and irrigation. No manures were applied in the experimental plots at any stage of growth of the plants. Identical field conditions were maintained in all the generations studied ($M_1-M_4$).

Germination and survival of plants

Percentage of germination of treated and control materials were recorded both in the laboratory at room temperature and in the field plots, but survival of plants was studied only in the field conditions. Growth of radicle following bursting of seed coat was taken into account for germination in petridishes; whereas in the field the
emergence of hypocotyl and cotyledon above the soil surface has been taken as an index for germination.

**Seedling growth**

Seedling growth was measured both in petriplates and field conditions from the M₁ samples.

In petriplates, seedling growth was measured from treated and control samples. Length of radicle and plumule was measured at random from 20 seedlings from each stock on a millimeter graph paper on the 10th and 15th day from the date of treatment. In field conditions, however, seedling growth was measured on the 30th day of sowing and the extent of injury was assessed by determining the relative reduction in growth in the treated plants as compared to controls.

**Morphological studies in M₁ and M₂ plants**

Morphological variations in the treated plants were studied in comparison with control during M₁ and M₂ generations. Seed germination and number of days required by the plants for producing the first flower were recorded at the seedling stage, while other morphological parameters were studied only after harvest. Plants were harvested after complete senescences.
Harvested plants were uniformly dried before recording the different morphological parameters.

Selfed seeds of individual M₁ plants were harvested separately. At random 50 seeds from each of the M₁ plants were sown separately in plant to row, germination of which was determined, and M₂ families were raised accordingly. Due to total failure of germination of M₁ seeds following 4 hours treatment with 0.75% and 1.0% EMS, no M₂ plants could be raised. Hence the data recorded from M₂ mutation lines following 4 hours treatment with 0.5% EMS only have been presented in tables.

Data were recorded from all the M₁ plants of the treated samples and 10 randomly selected plants from each M₂ lines. From each of the M₁ and M₂ generations, 100 control plants were studied. The parameters on which observation were made included plant height (only shoot lengths), number of primary and total branches, number of capsules per plant, capsule length per fruit, number of compartments, filled seeds per capsule and weight of 100 seeds.

Seed sterility was noted in the M₁ samples. Mean number of seeds per plant was determined from the surviving M₁ plant progenies in each treatment as well as in control and seed
sterility was represented as percentage of reduction in seed number in relation to control.

**Detection of mutant plants**

The plant progenies showing qualitative changes in stem, leaf and flower characters were scored in the $M_1$ generation from seedling stage to maturity and variations were determined from the percentage of $M_1$ plants survived.

Both viable and nonviable mutant plants (including chlorophyll mutation) from each of the $M_2$ lines were screened. The chlorophyll mutation types were classified after Blixt (1961) with slight modifications. Mutation frequency was estimated as per 100 $M_2$ plants (Gaul 1964). The efficiency and effectiveness of the two mutagens (X-ray and EMS) were compared by using the formulae suggested by Konzak et al. (1966). The efficiency was calculated as $Mf/L$, $Mf/I$ and $Mf/S$ and the effectiveness as $Mf/Ext$ or $Mf/KR$, where $Mf = M_2$ mutation frequency; $L = \text{percentage of lethality}; I = \text{percentage of injury}; S = \text{percentage of seed sterility}; C = \text{concentration of the mutagen}; t = \text{time of treatment}; KR = \text{radiation doses}$. 
Cytological studies

Mitotic and meiotic observations were made from the control and treated materials (M₁ generation). Besides, meiotic abnormalities were studied from the mutant plants.

Mitosis: Mitotic studies were made from the healthy root-tips of M₁ samples following X-irradiation and EHS treatment in the same procedure as adopted in case of control materials.

Frequency of chromosomal abnormalities was determined from total number of dividing cells and that of micronuclei was estimated from total resting cells. The percentage of total anomalies was, however, calculated from the frequency of total cells scored. Photomicrographs were taken from temporary preparations.

Meiosis: Flower buds of suitable size were collected and fixed in between 8 A.M. and 9 A.M. in propiono-alcohol (1:3) for 24 hours and finally preserved in 70% alcohol. Anthers were smeared in 1% propiono-carmine. Overstaining was avoided by carefully passing 45% acetic acid between the slide and the coverslip. Well scattered plates were obtained by alternate warming and mild pressure. Slides were temporarily sealed with paraffin and made permanent later by passing through ethanol-normal
butanol schedule and mounted in euparol. All observations were made from temporary slides. Meiotic abnormalities were calculated from total number of pollen mother cells (PMCs).

For gross chromosomal studies (M₁ samples) 3 to 4 randomly selected anthers from different flower buds were squashed per slide but for meiotic chromosome studies in the mutant plants single microsporophyll from each bud was squashed per slide. Photomicrographs were taken from suitable plates.

**Determination of pollen fertility**

The pollen grains of control, M₁ treated samples and that of the mutant plants were dusted in a drop of 1% aceto-carmine solution on a slide and coverglass slip was put on them. Pollen fertility was determined on the basis of stainability of pollen grain in aceto-carmine. Well stained pollen grains with regular shape were considered viable and empty or partially filled shrunken deformed pollen grains without any stain were considered as sterile.

**Studies on the M₂ mutants**

Morphological variations in the mutants were studied in comparison with control. The parameters recorded in the mutant plants were germination, survivality, plant height, number of
capsules per plant, capsule length per fruit, number of compartments per fruit, seed set per capsule (filled seed), 100 seed weight, seed size (length and thickness) and harvest index. Harvest index was calculated by using the formula:

\[
\text{Harvest Index (HI)} = \frac{\text{Economic yield}}{\text{Biological yield}} \times 100
\]

Seed size was measured by using diallel gauze. In addition to these observations, sterility of flower, capsule, pollen and seed was assessed in the mutant lines by using the following formulae:

\[
\text{Flower sterility} = \frac{\text{Total No. of capsule/plant}}{\text{Total No. of flower/plant}} \times 100
\]

\[
\text{Capsule sterility} = \frac{\text{Total No. of abortive capsules/plant}}{\text{Total No. of capsule/plant}} \times 100
\]

\[
\text{Seed sterility} = \frac{\text{Total No. of abortive seeds/plant}}{\text{Total No. of seeds/plant}} \times 100
\]

Biometrical approach

Analysis of variance, correlation and path coefficient analysis in control and mutant plants were studied. The true breeding mutant plants were sown in randomized block design with
three replications at the experimental plots during M₄ generation. In all cases, to avoid the border effect, control plants were grown along the border lines of the plots. A control set was also maintained under similar conditions. In each case, observations were made from 30 randomly selected plants. Observations on (i) plant height, (ii) total number of primary branches, (iii) total branches per plant, (iv) number of capsule per plant, (v) capsule length, (vi) number of compartments per capsule, (vii) filled seeds per capsule, (viii) total seeds per capsule, (ix) length and thickness of seeds, (x) seed yield per plant were recorded. All observations relating to fruit were made from the first capsule to avoid biasness. In addition to these observations, percentage of germination and survival, flower, capsule, pollen and seed sterility and harvest index were also noted. From these observations, the extent of variations between control and mutant lines growing under uniform environmental conditions were studied following analysis of variance.

The control plants, which were grown in randomized block design in the different generations (1979-80, 1980-81, 1981-82 and 1982-83), were subjected to analysis of variance for studying the extent of variations among the parameters during different generations.
The morphological parameters observed in the controls and mutant lines in $F_4$ generation were analysed to determine correlation, which would be helpful in demonstrating the association amongst the different morphological characteristics. Components of correlations were computed through path analysis following Dewey and Lu (1969) using five characters, viz., plant height, number of primary branches, total capsule per plant, number of total seeds per capsule and seed yield per plant.

Chlorophyll estimation

Chlorophyll pigment was estimated from the leaf tissues of control and five different types of chlorophyll mutant plants following the methods of Arnon (1949). For chlorophyll extraction, samples of 0.2 grams of fresh lamina (using leaves of similar maturity) were put into glass-stoppered conical flask containing 25 ml of 80% acetone and kept overnight at 4°C to dissolve the pigments. The solution was filtered through Whatman No. 1 filter paper pre-soaked with 80% acetone. The total volume of the extract was made up to 100 ml by the addition of required quantity of 80% acetone in a volumetric flask.

For densitometric reading, 5 ml of the extract was
transferred to a 50 ml volumetric flask and made upto the volume with 80% acetone. Readings of Optical Density (O.D.) values of this solution were recorded in a Spekol Colorimeter Carlzeiss (Jener) at 663 nm and 645 nm for chlorophyll a and chlorophyll b respectively against 80% acetone.

Estimation of chlorophyll a, b and total chlorophyll was made by using the following formulae:

mg of chlorophyll "a"/gm of tissue

\[ \text{mg of chlorophyll } "a"/\text{gm of tissue} = \frac{12.7 \times (D_{663}) - 2.69 \times (D_{645})}{100 \times W} \]

chlorophyll "b"

\[ \text{chlorophyll } "b" = \frac{22.9 \times (D_{645}) - 4.68 \times (D_{663})}{100 \times W} \]

Total chlorophyll

\[ \text{Total chlorophyll} = \frac{30.2 \times (D_{645}) + 8.92 \times (D_{663})}{100 \times W} \]

Final quantitative determination of chlorophyll content was performed by averaging the values of all samples of the same category (three replicas) in each plant type.
Mode of inheritance in the mutant plants

The segregation pattern of mutant and normal phenotypes were studied from the F2 progeny and the goodness of fit of the data was analysed by means of $X^2$-test.

Estimation of seed protein content in control and mutant plants

The seed samples were defatted by using petroleum ether (40-60°C m.p) for overnight at 16°C. Defatted seed samples were weighed and grounded in a pre-cooled mortar and pestle with 0.2 M Na-phosphate buffer at 6.8 pH. The homogenate was centrifuged at 100 Xg in the cold (4°C) for 10 minutes in a Sorbel centrifuge. The supernatant was treated with an equal volume of chilled 40% TCA and kept in a refrigerator for overnight. The precipitate formed was centrifuged at 6000 Xg for 15 minutes and the pellet was washed several times with demineralized H2O to free it from TCA. The washed powdery precipitate was dissolved in phosphate buffer to necessary dilutions.

Protein was estimated by the method of Lowry et al. (1951). To 0.5 ml of the protein solution, 2.5 ml of a mixture of reagent A (2% Na2CO3 in 0.1 N NaOH) and reagent B (0.5% CuSO4·5H2O in 1% Na-K-tartarate) mixed in the ratio
of 50:1 were added and allowed to stand for minutes. Then 0.25 ml of Folin-Ciocalteu reagent (Prepared according to the procedure outlined by Spies 1955) was added and left for one hour. The optical density of the blue colour developed was recorded in a "Carlzeiss" Spekol Colorimeter at 660 nm. Protein content was estimated by referring to a standard curve prepared with bovine serum albumin fraction V (Nutritional Biochemical Corporation, USA).

Quantitative estimation of oil content

Fatty oil has been extracted from the seeds of control and two mutant plants. Sun dried powdered seeds (10 gms with two replica each) were used for extraction of fatty oil using petroleum ether (60-80 m.p.) in a Soxhlet apparatus for 6-7 hours. The extracted oil was filtered and the excess petroleum ether was removed following drying in a hot water bath at 40°C for 12 hours. The quantity of oil was determined in percentage of dry weight of seeds.