3.1 Materials

Taxonomically diverse plant species suitable for cytological analysis, namely *Vicia faba* (faba bean) belonging to the family Leguminosae, sub family Fabaceae (family Leguminosae) and *A. cepa* L. syn. *aggregatum* (onion or chives) belonging to Liliaceae family, were selected as the experimental material.

Seeds of *V. faba* var. VH 82-1 were obtained from the Medicinal and Aromatic Plant Section, Department of Plant breeding, Haryana Agricultural University, Hisar, India and bulbs of *A. cepa* L. syn. *aggregatum* var. CO4 were obtained from the College of Horticulture, Tamilnadu Agricultural University, Coimbatore, India. The *V. faba* seeds were stored at low temperature (4°C) and *A. cepa* bulbs were stored in cotton bags at room temperature till use.

3.2 Germination of seeds and sprouting of bulbs

Seeds of *V. faba* wrapped in cotton cloth were soaked in water for 12 hours. The soaked seeds were then decoated and placed in petriplates on moist filter paper. The petriplates were incubated at 25±2°C for germination. From mature bulbs, the outer loose dry scale leaves and old roots were removed to facilitate rooting. For emergence of sprouts, the bulbs were placed in moist soil.

3.3 Mutagens used

Five mutagens - three physical mutagens - γ-radiation, Laser radiation and UV radiation and two chemical mutagens - Ethyl Methane Sulponate and Hydroxyl amine were used for the present study.
3.4 Irradiation of samples by physical mutagens

3.4.1 \( \gamma \)-irradiation

Gamma irradiation was done at the Bhabha Atomic Research, Centre, Mumbai, India. Dry seeds of \textit{V. faba} and bulbs of \textit{A. cepa} were irradiated at doses 2, 5, 10, 15 and 20kR. Treated seeds were stored in fridge at 4\(^\circ\)C and bulbs in a cotton bag at room temperature till use.

3.4.2 Laser irradiation

Laser irradiation was done at the International School of Photonics, Cochin University of Science and Technology, Cochin, India. Argon ion laser source (Spectra Physics Model - 171) was used for the irradiation \textit{V. faba} seeds were soaked over night in tap water and decoated before irradiation. Old roots and outermost old scale leaves were manually removed in the case of \textit{A. cepa} bulbs and were planted in moist sand over night to encourage root and shoot initiation before irradiation.

The embryo portion of the seeds and the apical bud portion of the bulbs were exposed to 476, 488 and 514 nm excitations from Argon ion laser source. In 514 nm various power levels - 200, 400, 600 and 800 mW with different power densities 2.25, 4.49, 6.74 and 8.89 mW cm\(^2\) and different exposure times - 10, 20, 30 and 40 minutes exposures were tested. In 476 and 488 nm excitations the power levels 200 and 400 mW with the corresponding power densities and exposure times of 10, 20, 30 and 40 min were tested. The Laser beam size was adjusted using proper optical elements so as to get uniform illumination in the sample container.
3.4.3 UV irradiation

The UV irradiation was done on materials subjected to pre-germination process done as in the case of laser irradiation. The embryo and apical bud portion of the materials were exposed to 30 Watt UV lamp attached to laminar air flow for 1, 2 and 3 hours at a distance of 5 cm from the source.

3.5 Chemical mutagens treatment of materials

The samples for chemical treatments were also subjected to pre-germination/sprouting processes as in the case of the aforesaid methods. The samples were then soaked in 0.2, 0.4, 0.6, 0.8 and 1% of Ethyl Methane Sulphonate solutions for 30 minutes and the same concentrations of Hydroxyl amine solutions for 1 hour. The solutions were prepared in distilled water.

3.6 Sprouting/germination of treated material

Immediately after the various treatments, the onion bulbs were replanted in moist sand and the faba bean germinated on moist filter paper in petridishes at 25±2°C. In the case of γ-irradiated samples however, the materials were kept for germination only just prior to collection of root tips for analysis.

3.7 Collection of root tips for mitotic studies

Onion root tips of length 1-2 cm were collected between 8 and 9 A.M on the 3rd day while in faba bean root tips of the same size were collected between 10 and 11 A.M after 3-5 days of treatments.
3.7.1 Pre-treatment of root tips

The root tips thus collected were pre-treated in 0.04% colchicine solution for about 3 hours at room temperature.

3.7.2 Fixation of pre-treated root tips

The root tips were washed twice with distilled water for removing the pre-treating agent and were fixed in a mixture of 3 parts of absolute alcohol and 1 part of glacial acetic acid. Fixative was prepared fresh every time. Root tips could be kept in the fixative upto 15 days in the refrigerator. Some of the root tips were fixed directly without colchicine pre-treatment for anaphase study.

3.7.3 Preparation of root tip squashes

From the fixative, the roots were transferred to distilled water and were washed twice. They were then hydrolysed in 1N Hydrochloric acid at about 60°C for a few seconds. After hydrolysis, the root tips were washed twice in distilled water and then transferred to distilled water.

On a clean slide, the tips were separated from the rest of the root and crushed in a drop of 2% acetocarmine with the flat end of a rod and squashed under a coverslip. The pressure was applied under several thickness of blotting paper. Sideways movement of coverslip was avoided in order to prevent the rolling of cells.

3.8 Photomicrography

Photographs were taken from temporary slides. Camera of the model Nikon F-601M was used for taking photographs Film ORWO NP 22 and
NOVA FP4 with the speed of ASA (ISO) 125 DIN 22 was used. All the photographs were taken under oil immersion lens.

3.9 Scoring of slides

For scoring of cytological aberration, temporary slides were used. At least 6 slides were prepared from actively dividing root tips in each dose and 15-20 fields (approximately 5,000-10,000 cells) were scored. Different structural changes of chromosomes were scored at metaphase and anaphase. Micronuclei were scored at anaphase/telophase. Savage’s (1975) classification of various types of chromosomal aberration was used for scoring the aberration. Mitotic index (M.I.) was calculated as below:

\[
\text{M.I.} = \frac{\text{Number of dividing cells}}{\text{Total no. of cells}} \times 100
\]

3.10 Meiotic studies

Selected treatments of the mutagens were used for the meiotic studies. The treatments included: Laser irradiation at two doses (viz. 10 min exposures at power density 200 mW and 30 min exposure at power density 400 mW) in each wavelength of 476 nm, 488 nm and 514 nm, UV exposures of 1 hour and 3 hour, γ-irradiation at 2, 5, 10, 15 and 20 kR, EMS treatment at doses 0.2% and 1% and Hydroxyl amine treatment at doses 0.2% and 1%.

Young flower buds were collected between 8 & 10 A.M. and the flower buds were fixed in absolute ethyl alcohol : acetic acid (3:1) mixture for at least 24 hours. The fixed flower buds were stored in fridge at ~ 4°C for subsequent use. Anthers were squashed in 1% iron-acetocarmine. Temporary slides were used for scoring. Several slides were prepared in each treatment.
Photomicrographs were taken from temporary slides as in the case of mitotic studies.

3.11 Pollen stainability

Pollen stainability was determined using 1% iron acetocarmine smear technique. At least 3 smears were prepared in each dose and many fields in each smear were counted. The mean of these values were used for determining the percentage of sterility of pollen grains.

3.12 Germination/Sprouting index, Growth and Yield studies

Germination/sprouting index growth and yield was monitored in selected treatments of the mutagens used in this study. The treatments included: laser irradiation at two doses (viz. 10 min exposure at power density 200 mW and 30 min exposure at power density 400 mW) in each wavelength of 476 nm, 488 nm and 514 nm, UV exposure of 1 hour and 3 hour, γ-irradiations at 2, 5, 10, 15 and 20 kR, EMS treatment at doses 0.2% and 1% and Hydroxylamine treatment at doses 0.2% and 1%.

25 seeds of *V. faba* and 20 bulbs of *A. cepa* in each treatment were sown in the field at the Indian Agricultural Reserch Institute Regional Station, Wellington, The Nilgiris, Tamilnadu, India at a spacing of 20 cm between plants and 50 cm between rows.

The germination/sprouting index was computed using the equation:

\[
\frac{\text{No of seeds(bulbs) showing seedling(sprouting)}}{\text{Total No. of seeds(bulbs)}}
\]
Growth was monitored as the change in plant height during the crop duration.

Yield was calculated on the basis of the equation:

\[
\frac{\text{Total weight of seeds (bulbs) in each treatment}}{\text{Total number of plants in each treatment}}
\]

Two repeats of each treatment were done.

### 3.13 Estimation of soluble protein content and assay for enzyme activity

The estimation of soluble protein content and assays for enzyme activity of protease, amylase, peroxidase and catalase were done in both plant species in selected mutagen treatments. The treatments included: laser irradiation at two doses (Viz. 10 min exposure at power density 200 mW and 30 min exposure at power density 400 mW) in each wavelength of 476 nm, 488 nm and 514 nm, \( \gamma \)-irradiation at 2.5, 10, 15 and 20 kR and EMS treatment at doses 0.2\% and 1\%.

The estimation of protein content and activity of various enzymes were assayed separately in embryo and cotyledon in *V. faba* while in *A. cepa* it was assayed separately in the inner true leaves with apical bud and in outer fleshy scale leaves. Amylase assay was done on second day and fourth day of germination, protease on third day and sixth day and peroxidase and catalase on fifth day of germination in *V. faba* while in *A. cepa* estimation of protein content and assay of these enzymes were done on second day and fourth day of sprouting. Peroxidase activity in *A. cepa* bulbs treated with EMS was done only on fourth day of sprouting.
Germinated seeds of *V. faba* and sprouted bulbs of *A. cepa* were harvested on specified days. The seeds were dissected into embryo and cotyledon and bulbs into inner true leaves with apical bud and outer fleshy scale leaves on aluminium foil placed on ice. The two samples were then cut into pieces with the help of scissors, weighed and homogenised using suitable buffer in a precooled mortar and pestle. The slurry thus obtained was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant used as enzyme source.

### 3.13.2 Protein estimation

Protein content was estimated by the method of Lowry *et al.*, (1951). 0.2 ml of test sample was made up of 1.0 ml with distilled water to which 2 ml protein reagent (prepared by mixing 0.1 N sodium hydroxide containing 2% sodium carbonate, 1% copper sulphate and 2% potassium sodium tartate in the ratio 100:1:1) was added. After 10 min incubation, 0.5 ml Folin's reagent (1:2 dilution) was added and the samples incubated for 30 min at room temperature. The blue colour developed by the reduction of phosphomolydbic - phosphotungstic component in the Folin - Ciocalteau reagent by the aminoacids tyrosine and tryptophan present in protein was measured in a spectrophotometer at 660 nm. Concentration of protein was determined from a standard graph prepared using Bovine Serum Albumin.

### 3.13.3 Assay for Protease activity

Protease activity was assayed by a modification of the method of Dubey (1982). Casein according to Hammersten is used as substrate for protease. 1% casein solution was prepared in 0.1 M phosphate buffer (pH 7.6)
by heating for about 15 min in a boiling water bath until casein was dissolved. It was then diluted to 100 ml. Enzyme solution was prepared by extracting 150 mg sample in 1 ml 0.1 M phosphate buffer (pH 7). 0.1 ml substrate solution and 0.2 ml enzyme solutions were pipetted into test tubes. Control contained only substrate solution. The reaction mixture was incubated for one hour in a water bath at 37°C. Then 0.6 ml 5% TCA was added to arrest reaction and kept for half an hour. The mixture was centrifuged at 10,000 rpm for 10 min. The TCA soluble peptide fragments are measured at 660 nm by the method of Lowry et al., (1951). One unit of protease is expressed as micrograms of peptide fragments formed in 60 min incubation time and specific activity is expressed as activity/mg protein.

3.13.4 Assay for Amylase activity

Amylase assay was done by a method described by Sadasivam and Manickam (1992). 200 mg sample was extracted in 1.0 ml 66 mM phosphate buffer (pH 7) containing 0.5 M NaCl. The pellet was extracted with phosphate buffer containing 0.5% mercaptoethanol. The supernatants of two extractions were collected and mixed. To 1.0 ml 1% starch solution (prepared in acetate buffer, pH 4.7) 0.1 ml enzyme solution, diluted to 1.0 ml with phosphate buffer was added. The mixture was incubated at 27°C for 15 min. The reaction was stopped by addition of 2 ml dinitrosalicylic acid reagent. The solution was heated in boiling water bath for 5 min after which 1 ml 40% sodium potassium tartrate was added. After cooling the tubes, 6 ml of distilled water was added.

Absorbancy was read in a spectrophotometer at 560 nm. Concentrations were measured from the standard graph prepared with 0-
1000 μg maltose. One unit of amylase is expressed as μg of maltose produced during 15 min incubation with 1% starch and specific activity as activity per mg protein.

### 3.13.5 Assay for Peroxidase activity

Assay for peroxidase was done by a method described by Sadasivam and Manickam (1992). Guaiacol was used as substrate for the assay.

\[
\text{Guaiacol} + \text{H}_2\text{O}_2 \rightarrow \text{oxidised guaiacol} + 2\text{H}_2\text{O}
\]

The rate of formation of guaiacol dehydrogenation product is a measure of peroxidase activity and can be assayed spectrophotometrically at 436 nm.

1 g of fresh tissue was ground in 0.5 ml of 0.1 M phosphate buffer (pH 7) and the slurry centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used as enzyme source. 3 ml of 0.1 M phosphate buffer, 0.05 ml of 20 mM guaiacol solution, 0.1 ml of enzyme extract and 0.03 ml of 12.3 mM H₂O₂ solution was pipetted into a cuvette (H₂O₂ solution was freshly prepared by diluting 0.14 ml 30% H₂O₂ to 100 ml with distilled water), mixed and placed in a spectrophotometer, was adjusted to wavelength 436 nm. A stop watch was started and the time required to increase optical density by 0.1 is noted.

Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under conditions specified is 6.36 per micromole, the enzyme activity per litre of extract was calculated as below.

\[
\text{Enzyme activity (units/litre)} = \frac{3.18 \times 0.1 \times 100}{6.39 \times 1 \times \Delta t \times 0.1} = \frac{500}{\Delta t}
\]

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1.6 Assay for Catalase activity

Catalase assay was done by permanganate titration method. Catalase decomposes hydrogen peroxide to water and oxygen.

1g of tissue was extracted in 0.5 ml of 0.1 M phosphate buffer (pH 7). 15 ml of 0.01 N hydrogen peroxide was placed in an Erlenmeyer flask. 0.1 ml of catalase solution was added to it, while simultaneously starting a stop watch. After two min, 2 ml of the solution was withdrawn and added to an excess of 2% sulphuric acid for titration with 0.01 N potassium permanganate. The quantity $x_1$ is determined. Then 2 ml of the solution was again withdrawn after 4 min and titrated as above to determine the quantity $x_2$.

The kinetics of hydrogen peroxide disappearance closely follow first order kinetics and the velocity constant ($K$) is calculated.

$$K = 2.3 \log \frac{x_1}{x_2}$$

The units are sec$^{-1}$. One unit is taken as the amount of enzyme which liberates half the peroxide oxygen from a hydrogen peroxide solution of any concentration in 100 sec at 25°C. The unit of enzyme activity is therefore related to the half life ($\tau$) of the first order reaction for $\tau$ the following formula is valid.

$$\tau = \frac{\ln 2}{K} = 0.693 \frac{1}{K}$$

The relationship between observed half life time $\tau$ observed and enzyme activity is,

$$1 \text{ unit} = \frac{100}{\tau \text{ observed}} = \frac{K \text{ observed}}{6.93 \times 10^{-3}}$$

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