3. MATERIALS AND METHODS

3.1. Transfer of specific desirable characters for the improvement of chillies

3.1.1. Details of seed materials: The recipient parents of the present study consists of five South Indian popular elite chilli varieties namely Bhagyalakshmi (G-4), Kovilpatti (K-2), Byadagi, Madurai-1 (MDU-1) and Coimbatore-2 (CO-2). The seed materials of G-4, K-2, MDU-1 and CO-2 were procured from Tamil Nadu Agricultural University, Coimbatore and seeds of Byadagi were procured from University of Agricultural Sciences, Dharwad, Karnataka. The details of the seed materials are given in Table 1. The donor parents contributing various specific desirable genes responsible for mechanical easy harvest includes (i) KAU-cluster - a popular cluster cultivar carrying specific genes for compactness ('cpt'), upright fruit character ('up'), cluster fruits ('cl'), and destalkness ('dst'), (ii) Kanthari local - a popular chilli cultivar characterized with small fruits with upright fruit type - belonging to Capsicum frutescens, carrying the gene for upright fruit ('up'), (iii) Ujwala - a chilli cultivar with upright and cluster fruit type (carrying the genes 'up' and 'cl'), and (iv) Dharwad cluster - a long pendulous cluster fruit bearing type carrying the gene 'cl' for cluster fruits.

3.1.2. Place of study: The work was conducted at Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India. The place, Coimbatore is having a congenial agroclimatic condition for hot chilli cultivation throughout the year. The major hot chilli varieties, adopted for this region are G-4, K-2, MDU-1 and CO-2.

3.1.3. Methods used for gene transfer: Four specific desirable genes namely 'cpt' (compactness), 'up' (upright fruit), 'cl' (cluster fruit) and 'dst' (destalkness), present either singly or in combination in four hot chilli stocks, responsible for easy mechanical harvest were transferred to five Indian popular hot chilli varieties namely G-4, K-2, Byadagi, MDU-1 and CO-2. All the genes under transfer are found recessive in nature, therefore, these genes were transferred by simple backcrossing followed by selection.
Table 1. Details of parentage, year of release, name of the organisation developed and main characteristic features and remarks regarding the chilli parents used.

<table>
<thead>
<tr>
<th>Name of the chilli variety</th>
<th>Parentage</th>
<th>Year of release</th>
<th>Name of the Institute / University</th>
<th>Main characteristic features</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-4 (Bhagyalakshmi)</td>
<td>Selection from Thohian chillies from Srilanka</td>
<td>1972/1977**</td>
<td>Agricultural Research Station, Lam, Guntur, Andhra Pradesh, India</td>
<td>Released at State as well as National level. The leaves are narrow and dark green, fruits live green turning dark red on ripening. Seed content 38 to 40%. Fairly tolerant to disease and insects. Average yield (dry) 11.76 q/ha under rainfed and 50 q/ha under irrigation. Fruit size vary from 7 to 8 cm long and 3-3-5 cm girth.</td>
</tr>
<tr>
<td>K-2 (Kovilpatti-2)</td>
<td>Assam type B 70A X Sattur Samba</td>
<td>1975 / 1985**</td>
<td>Regional Agricultural Research Station, Kovilpatti, Tamil Nadu, India</td>
<td>Released at State and National level. Plant tall, semi spreading type with duration 210 days, pericarp thickness 0.19 mm, red fruit colour. 80-85 seeds with blunt tap. Capsaicin content 0.49 mg/g and average yield (dry) is 18 q/ha.</td>
</tr>
<tr>
<td>Byadagi</td>
<td>It is a local selection</td>
<td></td>
<td>Karnataka Agricultural University, Dharwad</td>
<td>Released for the state of Karnataka for Dharwad region as a rainfed crop. The plants grow to a height of 1m with a spread of 1m. Leaves are thin and light green in colour. It is a high branching type. Fruits attain deep red colour on maturity and develop wrinkles on the surface. Fruits are 12-15 cm long and thin but less pungent. Extensively cultivated in transition belt of Dharwad, Shimoga and Chitradurg districts. Average dry fruit yield 25 q/ha.</td>
</tr>
<tr>
<td>MDU-1 (Madurai-1)</td>
<td>A mutant variety of K1</td>
<td>1975</td>
<td>Agricultural college and Research Institute, Maduri, Tamil Nadu, India</td>
<td>Released at State level. Plant dwarf, compact, suited for high density planting. A closer spacing 30 X 30 cm recommended, bear fruits in clusters (4 to 9 / cluster), fruit is long (8 cm), red colour, 50 to 60 seeds/fruit, crop duration 210 days, high Capsaicin content, yield on average is19 q/ha.</td>
</tr>
<tr>
<td>CO-2 (Coimbatore-2)</td>
<td>Selected from gundu type</td>
<td>1977</td>
<td>Tamil Nadu Agriculture University, Coimbatore, India.</td>
<td>Released at State level. Fruit is small (2-4 cm long), dark red colour, 7-8 seeds/fruit, crop duration is 200 days. Average yield (dry) 23 q/ha.</td>
</tr>
</tbody>
</table>

** Year of national release
3.1.4. Production of F₁ hybrids and backcross progenies: All the F₁ hybrids obtained from the crosses between recipient parents and the donor parents contributing the four genes, were backcrossed with respective recurrent chilli varieties to obtain BC₁ hybrids. In the above cross, the F₁ hybrid was used as female parent and the recurrent parent was used as male parent. All the BC₁ hybrid plants were selfed and screening and selection was made in BC₁F₂ plants for characters, which are under transfer. Selected BC₁F₂ plants were again backcrossed twice with respective recurrent chilli varieties to produce BC₂ and BC₃ hybrids. Selfing was done in BC₃ to produce BC₃F₂. One line from each of the BC₁F₂ and BC₃F₂ were constituted carrying the desirable characters.

3.1.5. Agronomical performance of the constituted lines: The quantitative characters such as plant type, plant height, number of fruits per pedicel, number of fruits per plant, position of the fruits (upright or pendulous) and destalkness of the fruit were recorded in all the constituted lines. The lines recorded for plant type, plant height, fruit type, number of fruits per pedicel, number of fruits per plant etc. were the average of 20 randomly selected plants from all the constituted lines. These lines were grown in the field in two rows of 1 meter each. All the quantitative characters of the constituted lines were compared with their respective recurrent parents.

3.1.6. Confirmation of transfer of specific genes into commercial cultivars

A. Confirmation through morphological studies: The inheritance of morphological characters of the donor parents such as type of branching, fruiting habit, fruit orientation and destalkness were recorded and compared with the constituted lines.

B. Confirmation through genetical studies: (i) Inheritance studies - Selective lines of the constituted lines were crossed with the local variety (K-1), carrying erect plant type, pendulous solitary fruit type and strongly stalked pedicel, to produce F₁ hybrids. The hybrids were selfed as well as backcrossed with recurrent parent to produce F₂ and BC₁. F₁, F₂ and BC₁ populations were evaluated for inheritance of characters that are under study. Segregation pattern was recorded and Chi-square test was applied.
C. Confirmation through biochemical studies: The nuclear DNA content of recurrent parents and their constituted lines carrying various genes for desirable characters were estimated with the help of Vickers's M 85 Scanning Microdensitometer, and the mean and range values were compared. The staining procedure followed was after Reddy (1989).

3.1.7. Biometrical studies

(i) Analysis of variance - estimates of range, mean, GCV, PCV, heritability and genetic advance were carried out with 20 cross combinations (which include constituted lines).

(ii) Path analysis - genotypic correlation coefficients, phenotypic correlation coefficients, simple correlation coefficients and direct and indirect effects of characters on fruit yield were analysed through path analysis.

(iii) Statistical analysis

1. Analysis of variance for Randomized Complete Block Design was followed as suggested by Cochran and Cox (1959).

2. Variability studies

a. Genotypic and phenotypic variances

\[
\text{Genotypic variance } (\sigma^2_G) = \frac{\text{MSS}_i - \text{MSS}_e}{r}
\]

\[
\text{Phenotypic variance } (\sigma^2_G) = \sigma^2_G + \sigma^2_C
\]

\[
\text{Error variance } (\sigma^2_e) = \text{MSS}_e
\]

b. Heritability \((h^2)\)

Heritability in the broad sense was determined suggested by Hanson et al. (1956).

\[
h^2 = \frac{\sigma^2_G}{\sigma^2_P} \times 100
\]

c. Genetic advance (GA)

It was determined using the formula suggested by Johnson et al. (1955).
3.2. Induced mutations in chillies

3.2.1. Seed materials: The seed materials used in the present induced mutation experiment consisting of four diploid chilli varieties viz. Bhagyalakshmi (G-4), Kovilpatti (K-2), Byadagi and Coimbatore-2 (CO-2). The seed materials were procured from TNAU, Coimbatore, and from University of Agricultural Sciences, Dharwad, Karnataka.

3.2.2. Mutagens: One physical mutagen (gamma rays) and two chemical mutagens (EMS and MMS) used individually and in combination.

(i) Gamma rays: Gamma rays were secured from gamma chamber \(^{60}\text{CO}\) operating at voltage 30 kV with an intensity of 1000 r/min located at Sugarcane Breeding Institute, Coimbatore.

(ii) Ethyl Methane Sulphonate (EMS): EMS of MERCK, Germany was procured and aqueous solution of 0.5% was prepared and was used in the present study.

(ii) Methyl Methane Sulphonate (MMS): MMS of MERCK, Germany was procured and aqueous solution of 0.5% was prepared and was used in the present study.

3.2.3. Methods

(A) Treatments - Individual and combination treatments (physical and chemical) of the above were used. Dry seeds of all the four chilli varieties viz. G-4, K-2, Byadagi and CO-2 were irradiated with three doses of gamma irradiation viz. 15, 25 and 35kR. For EMS and MMS treatment, seeds were treated with 0.5% aqueous solution of EMS and MMS for 8, 12 and 16 hours duration, respectively. For combination treatments, combinations were prepared in such a way that higher doses of gamma rays were combined with lower duration of EMS and MMS and vice versa. Three combination treatments of each were given viz. 15kR+16h; 25kR+12h; 35kR+8h. Following EMS and MMS treatments, either individually or in combination, seeds were washed thoroughly in running water for
about 30 minutes and planted immediately in the field along with respective control. For each treatment, one hundred seeds were used.

(B) **Sowing plan:** Treated seeds of each treatment were sown separately in different rows along with respective controls. Spacing was maintained within the line and between the lines. *(30 cm x 45 cm)*

3.2.4. **Recording data:** (A) **M<sub>1</sub> generation:** The M<sub>1</sub> plants were studied to record the data on the following biological parameters.

(i) **Germination:** Germination was recorded after 15 days of sowing. The lethal dose 50 (LD 50) was calculated through regression lines in all the mutagenic treatments of all the four chilli varieties.

(ii) **Survival:** Survival of plants was counted at the time of maturity.

(iii) **Seedling height (cm):** The seedling height (cm) was recorded in twenty days old seedlings.

(iv) **Seedling injury:** Malformation of shoots, roots, seedling leaves and decolouration of leaves were recorded at seedling stage.

(v) **Stomatal studies:** Epidermal peelings of seedling leaves were used to study the stomatal index and frequency of abnormal stomata on both upper and lower surfaces of seedling leaves of control and treated materials.

(vi) **Chlorophyll variants:** Variation in the pigmentation in the seedling leaves including absence of chlorophyll pigments in certain regions of leaves, light green leaves, presence of xanthophyll, variegated leaves were recorded.

(vii) **Recovery Index:** The recovery index was calculated to find out the biological equivalents of the mutagens.

(viii) **Pollen sterility:** Pollen sterility was calculated by staining the pollen with aceto-carmine and unstained pollen grains were considered as sterile pollen. Co-efficient of
interaction ('K' values) was calculated in combined treatments for various biological parameters in M₁ generation.

(ix) **Meiotic studies:** Flower buds were collected from 20 randomly selected plants in M₁ generation from each treatment and fixed in freshly prepared Carnoy's fluid (6:3:1 - absolute alcohol : Chloroform : acetic acid) for 24 hours. Few drops of saturated solution of ferric chloride were added to the fixative to obtain better staining. Anthers were squashed in 2% aceto-carmine. Atleast 25 cells from each preparation were used to record data on various cytological parameters such as quadrivalents, trivalents, univalents, laggards, bridges, fragments and micronuclei. Meiosis was studied from both temporary and permanent preparations. Microphotographs were made from temporary slides. Slides were made permanent by using Euparol.

DMRT was applied to compare the mean values of control and mutagen treated population for both biological and cytological parameters.

(B) **M₂ generation:** All M₁ plants were bagged to avoid cross-pollination. Seeds from the first formed five fruits from each M₁ plant was collected separately and were sown as fruit to row progeny to raise M₂ generation. Spacing was maintained within and between the rows as in M₁ generation. The progeny rows were studied with respect to following parameters.

(i) **Chlorophyll mutants:** Chlorophyll mutants were observed and recorded at seedling stage after 15 days of sowing in all the four chilli varieties. The mutation frequency was calculated as per cent M₁ plants and per cent M₂ plants. The following formulae were used for calculating the mutation frequencies.

\[
\text{Mutations per cent } = \frac{\text{Total no. of segregating plant progenies}}{\text{Total no. of M₁ plant progenies}} \times 100
\]

\[
\text{Mutations per cent } (b) \quad \text{M₂ seedlings} = \frac{\text{Total no. mutant plants}}{\text{Total no. of M₂ plants}} \times 100
\]
The data on chlorophyll mutation frequency were also used to calculate the following mutagenic parameters.

(a) Mutagenic effectiveness
\[
\text{Mutagenic effectiveness} = \frac{M}{\text{Ct}} \text{ or } \frac{M}{kR} \text{ Or } \frac{M}{\text{Ct+kR}}
\]

Where,
- \( M \) = Chlorophyll mutation frequency in \( M_2 \) generation on \( M_2 \) plant basis
- \( C \) = Concentration of chemical mutagen
- \( T \) = Duration of mutagenic treatment
- \( kR \) = Dose of gamma rays

(b) Mutagenic efficiency
\[
\text{Mutagenic efficiency} = \frac{M}{L}
\]

Where,
- \( M \) = Chlorophyll mutation frequency in \( M_2 \) generation on \( M_2 \) plant basis
- \( L \) = Survival reduction of seedlings in \( M_1 \) generation

(c) Factor of effectiveness
\[
\text{Factor of effectiveness} = \frac{\text{No. of mutations (M}_1\text{ plant progeny)}}{\text{No. of seeds sown}} \times 100
\]

(d) Mutants per mutation
\[
\text{Mutants per mutation} = \frac{\text{No. of mutants from mutated plant progeny}}{\text{No. of plants from mutated plant progeny}} \times 100
\]

(e) Co-efficient of interaction ('K')
\[
\text{Co-efficient of interaction ('K')} = \frac{(a + b)}{(a) + (b)}
\]

Where
- \( a+b \) = The chlorophyll mutation frequency \((M_2)\) induced by the mutagens in combination
- \( a+b \) = The mutation frequency induced by the two mutagens when applied individually

Further,

If the value ‘K’ is one, the interaction is known as additive effect. If the value of ‘K’ is more than one or less than one, the interaction is known as positive synergistic and negative synergistic, respectively (Sharma, 1970).
Segregation pattern of each chlorophyll mutant type in a segregating row in M2 generation was recorded. Chi-square test was applied for testing the goodness of fit for segregation pattern.

(ii) **Morphological mutants:** Morphological mutants were isolated in M2 segregating rows in all the four chilli varieties. The mutants were selected on the basis of plant form, plant height, stem and fruit characters. Mutation frequency was calculated as percent M₁ plants and per cent M₂ plants. The individual frequency and spectrum of different morphological mutants were also calculated. Chi-square test was applied for testing the goodness of fit for segregation pattern of morphological mutants.

(iii) **Desirable mutants:** Various agronomical desirable mutants were isolated from M₂ segregating rows. These mutants were based on plant form, plant height, fruit number, number of seeds per fruit and days to flowering. Data on seven quantitative characters were recorded on the mutant plants. DMRT was applied to compare the mean values of various agronomic characters of the mutants with control.

(iv) **Induced variability:** Induced variability for eight different quantitative characters in M₂ generation were studied only in two chilli varieties G-4 and K-2. 10 randomly selected plants from each row were utilized to collect data on eight quantitative characters viz. plant height (cm), number of primary branches/plant, days to flowering, number of fruits per plant, fruit length (cm), fruit girth, harvest index and dry fruit yield (g)/plant. In control, one hundred plants were used to collect data on various agronomic characters. Estimates of mean, range, standard deviation, co-efficient of variation, heritability and genetic advance as percent of mean were calculated for each character from each treatment. DMRT was applied to test the means of mutagenic material with that of control.

(C) **M₃ generation**

(i) **Chlorophyll and morphological mutants:** Irrespective of the cultivar and treatment, seeds from individual plants in a segregating row (one line for each mutant
type) in $M_2$ generation were harvested separately, and were sown as plant to row to raise $M_3$ generation. Data on segregating pattern of chlorophyll and morphological mutants were recorded within the row and between the rows. Chi-square test was applied to test the goodness of fit for segregation pattern.

(ii) Desirable mutants: Seeds collected from each desirable mutant plant in $M_2$ generation were separately harvested and were sown as plant progenies in $M_3$ generation to study the breeding behaviour and their performance. Mutant plants, which did not breed true, were rejected and individual plant selections were again executed within the true breeding lines. Mean data on seven quantitative characters were recorded on the mutant plants along with the respective control. Mean comparisons were made by DMRT.

(iii) Induced variability: Induced variability for different quantitative characters were studied in two chilli varieties G-2 and K-2. A total of 100 plants were randomly selected from $M_2$ generation from each mutagenic treatment. The selected plants were sown in the field to raise plant progenies to raise $M_3$ generation. Data on eight quantitative characters were recorded on 10 randomly selected $M_3$ plants from each row. In control, one hundred plants were used to collect the above data. Mean, SD, co-efficient of variation, heritability and genetic advance was calculated for all the characters as in $M_2$ generation. DMRT was applied to test the means of mutagenic material with that of control.

3.3. Induction of autopolyplody in chilli

With a view to induce autotetraploidy, two chilli species were selected viz. *Capsicum annum* var. *California Wonder* and *Capsicum frutescens* var. *Kanthari Local*. Both the chilli lines are true breeding diploid species ($2n=2x=24$). Seeds were germinated in flowerpots. 0.1 to 0.5% aqueous solution of colchicine was administered to the seeds as well as to the seedlings. Three sets of each seed and seedlings were made and treated for three duration's viz. 16h, 20h and 24h, respectively. Young flower
buds of appropriate size were fixed in freshly prepared 1:3 aceto-alcohol mixture, transferred to 70% alcohol after 24h, and stored in the refrigerator until 2% aceto-carmine smears were made. A total of 25 cells from each preparation were used to record data on cytological parameters such as quadrivalents, bivalents, univalents, laggards, bridges, fragments and micronuclei. DMRT was applied to compare the mean values of cytological parameters of control and induced tetraploid plants. Estimation of capsaicin content and colouring matter (PPM) were done from the diploid and tetraploid chilli fruits.

3.3.1. Estimation of capsaicin content: Capsaicin content was measured by following the procedure of Quagliotti and Ottaviano (1971). Sun dried red chillies were oven-dried, ground to a fine powder and analysed for their capsaicin content. The colour was developed with phospho-molybdic acid in acetone extracts of chilli powder and it turned turbid after one hour. The turbidity was removed by quick filtration and subsequent centrifugation. The absorbency of clear solution was measured at 650 nm. It was compared with standard curve drawn with pure capsaicin.

3.3.2. Estimation of total colouring matter (PPM): Sun dried ripe chilli fruits were oven-dried, ground to fine powder and used to estimate total carotenoid pigment. The colour pigment extracted in water saturated n-butyl alcohol was measured at 435.8 nm after quick filtration and was expressed as total carotenoid pigments in PPM (AOAC, 1980).

3.4. Assessment of phylogenetic relationships in Capsicum through seed protein analysis

Protein extracts were prepared from fully ripened seeds harvested from greenhouse or field grown plants of 30 different Capsicum lines including domesticated species, wild species, commercial cultivars, exotic cultures, sweet pepper lines and local cultivars.

The machine used for the present study was SE-250 mini vertical gel electrophoresis unit and the unit holds two 10 X 8 cm gel sand witches (gel plate size 10
X 8 cm and approximate gel size is about 8 X 7 cm). Employing the polyacrylamide gel Electrophoretic technique outlined by Davis (1964) and Ornstein and Davis (1964) the soluble protein pattern was studied. Extraction was done by grinding the seeds in few drops of 0.01 M Tris HCl buffer (pH 7.5) using a chilled mortar and pestle. The extracts were centrifuged at 8000 rpm at 15 minutes in 4°C. The protein portion was separated and stored at 20°C until used. The crude protein extracts were analysed by slab gel electrophoresis using 11.5% polyacrylamide gel at a running pH 9.5 in Tris glycine buffer. At this pH, the proteins migrate towards the anode, permitting the use of Bromophenol blue as a tracking dye to mark the moving protein front. 0.5 mg of the protein sample dissolved in 0.1 ml of the stacking gel solution was applied to each column, with a current of 3 mA per column, about one hour of electrophoretic time was required for the tracking dye to travel the required distance of 4.5 cm marked on the glass tube.

At the end of electrophoresis, the slab gel were removed with a fine stream of water and immediately stained with 0.25% Coomassie brilliant blue in a solution of HO AC-MCOH.H2O (2:3:15) for 1 hour to locate the protein bands and destained with the same solution without the dye. The densitographs of the spectrum of soluble protein were prepared by scanning the gels on a Schimadzu UV-VIS double beam 160A Spectrophotometer. Electrophorograms were prepared on the basis of protein mobility and density expressed in ‘Rf’ values. The ‘Rf’ values for each band was computed from the mean of observations obtained from 5 independent Electrophoretic runs and 2 separate extraction’s. The percentage similarities between different pairs of species and varieties were calculated. The group affinity (GA) and Isolation values (IV) were calculated.

3.5. Induction of haploids in Capsicum through in vitro androgenesis

The experimental material comprised of two chilli species of Capsicum. Viz. C. annuum L var. grossum (sweet pepper) and C. frutescens L (hot pepper) The methods involved in the preparation of nutrient media, various cultural operations like preparation
and inoculation of anthers, callus induction, sub-culturing the callus, multiple shoot formation are presented below.

(i) **Preparation of medium:** Modified Murashige & Skoog (1962) and Linsmaier & Skoog (1965) medium were used. Media preparation includes preparation of stock solutions of various chemicals and their mixing in a proper sequence with desired concentrations.

(ii) **Preparation of stock solution:** The constituents of media were categorized into different stock solutions viz. I, II, III, IV, V, VI and VII depending on their solubility. The solution '1' includes ammonium nitrate (NH4NO3) and the solution '2' includes potassium nitrate (KNO3). I and II solutions were prepared freshly at the time of media preparation. The remaining stock solutions were prepared by weighing the required amounts of chemicals and dissolving them each in 200 ml of double distilled water. To prepare stock solution, Na2 EDTA was mixed with distilled water and slightly heated, then it was mixed with Fe2SO4.2H2O and thoroughly stirred to get more stable EDTA.

(iii) **Plant growth regulators:** (a) BAP and Kinetin: 200 PPM of the Kin/BAP stock solution was prepared by dissolving 20 mg of Kin/BAP in a few drops of 1N HCl and made up the solution to a final volume of 200 ml with double distilled water. (b) 2,4-D - 200 PPM of 2,4-D stock solution was prepared by dissolving 20 mg of 2,4-D in a few drops of ethanol and made up the solution to a final volume of 200 ml with double distilled water. (c) IAA and NAA - 200 PPM of IAA/NAA stock solutions were prepared by dissolving 20 mg of IAA/NAA in a few drops of 1N NaOH and made up the solution to a final volume of 200 ml with double distilled water. All the stock solutions were stored in refrigerator. From these stocks, approximate amount of stock solutions was taken to prepare required amount of media. All stock solutions were mixed in sequence and made up the final required volume by adding double distilled water. The desired concentration of hormones were made as:
Amount of hormonal solution required = \frac{\text{Conc. required} \times \text{Volume made}}{\text{Conc. of stocks}}

(iv) Cultural operations and conditions: Capsicum buds containing green anthers with a few violet tinge were superficially disinfected with 0.5% mercuric chloride for 10-15 min and washed 3 times with sterile water. Heat (35°C at 10 to 15 min.) as well as cold treatments (+4°C in dark chamber for 3 to 7 days) were given separately to the isolated buds. The anthers were aseptically cultured on modified L.S medium with different hormonal combinations.

Chilli anthers were inoculated on LS medium with different hormonal combinations:

(v) Sub-culturing: Callus induction was observed after 25 to 30 days of inoculation of the anthers. Callus was sub-cultured regularly at four week intervals on the medium with the hormonal combinations:

- MS + 2.0 mg L⁻¹ 2,4-D + 1.0 mg L⁻¹ KIN
- MS + 2.0 mg L⁻¹ 2,4-D + 1.0 mg L⁻¹ BAP

Four-week-old primary embryo callus was transferred to MS medium with the following hormonal combinations for regeneration.

- MS + 0.5 mg L⁻¹ IAA + 2.0 mg L⁻¹ KIN
- MS + 0.5 mg L⁻¹ IAA + 4.0 mg L⁻¹ KIN

(vi) Growth measurements: The effect of different hormones in MS basal media in the growth of anthers in culture was analysed by taking the following parameters.

(vii) Growth and differentiation: The differences in two species of chillies were recorded in terms of the size of anthers exhibiting its growth in culture during initiation of callus, development and regeneration of plantlets. The differences were measured in terms of frequencies of callus induction, shoot formation, root formation, callus + shoot formation, callus + root formation and regeneration and morphological judgment in terms of good, moderate, poor systems of grading was used to differentiate the two different chilli types.
(viii) **Callusing frequencies**: Cultures were scored for the frequency of callus induction at the end of fourth week. This callusing frequency was measured as the ratio of the number of sterile anthers initiated to the total number of anthers inoculated and was expressed in percentage. The frequency of callusing and regeneration of anthers and genotypes were recorded. The frequency was compared as the ratio between the number of anthers responded to callus initiation or regeneration to that of the total number of anthers inoculated and was expressed in percentage i.e.

\[
\text{Callus initiation / Regeneration \%} = \frac{\text{No. of explants initiated}}{\text{No. of plants inoculated}} \times 100
\]