Material and Methods
3. MATERIAL AND METHODS

Plants were grown and maintained in the experimental Garden of the Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad, Karnataka, India (Fig. 1) which is located at 15° 26’ N, latitude and 75° 07’ E longitude and at an altitude of 678 meters above mean sea level (MSL) in the Northern Transitional Agro-climatic zone (Zone VIII). The meteorological data has been given in Appendix I.

Experiment 1: Molecular diversity analysis and variability studies

3.1 Experimental materials used and methods followed

Forty five accessions of *Capsicum annuum* L. collected from chilli Research Station, Devihsour, Horticultural University, Bagalkot and maintained at UAS, Dharwad (Table 2) were used as source material for genetic diversity and variability studies. The germplasm was sown in seed beds in November 2007 and transplanted during December in the main field in randomized block design with two replications consisting of single row of 8 plants for each entry. A row to row and plant to plant spacing of 60 cm was followed and the crop was raised as per the recommended package of practices.

3.1.1 DNA Analysis

3.1.1.1 RAPD fingerprinting

3.1.1.1.1 Isolation of Genomic DNA

Genomic DNA was extracted from young leaves according to the modified CTAB method described by Doyle and Doyle (1990). One gram of fresh, young, deveined leaves was homogenized in pre-chilled mortar and pestle in liquid nitrogen. The homogenate powder was incubated in 10 ml preheated CTAB isolation buffer [2% w/v CTAB (Cetyl Trimethyl Ammonium Bromide) (GeNei™, Bangalore, India), 1.4M NaCl, 0.2% 2-mercaptoethanol, 20mM EDTA, 100mM Tris HCl (pH 8.0), 2% PVP] in serological water bath at 60 °C for 30 minutes with occasional gentle swirling. The homogenate was mixed with 10 ml of Chloroform-Isoamylalcohol (24:1)
Table 2: Chilli accessions used for genetic variability and molecular diversity analysis

<table>
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<tbody>
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<td>Byadgi Dabbi</td>
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<td>VN2</td>
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<td>19.</td>
<td>DCA19</td>
<td>42.</td>
<td>Phule Jyoti</td>
</tr>
<tr>
<td>20.</td>
<td>DCA20</td>
<td>43.</td>
<td>Nooji</td>
</tr>
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<td>21.</td>
<td>DCA21</td>
<td>44.</td>
<td>Yellow capsicum</td>
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<tr>
<td>22.</td>
<td>DCA22</td>
<td>45.</td>
<td>Red capsicum</td>
</tr>
<tr>
<td>23.</td>
<td>DCA23</td>
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</table>

36
and mixed vigorously. The mixture was centrifuged at 10,000 rpm, for 30 minutes at 20°C. The aqueous supernatant was transferred with a wide bore pipette to a new centrifuge tube. To the above extract 2/3 volumes of cold isopropanol was added, mixed gently to precipitate the nucleic acids. Precipitated nucleic acids were gently spooled out with hooked pasture pipette, transferred to 10 to 20 ml of wash buffer (76% Ethanol and 10 mM Ammonium acetate) and the spool was washed for 20 minutes in wash buffer. DNA was air dried and resuspended in 1 ml of TE buffer [10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. After complete dissolution, the nucleic acids were incubated with RNase-A (final concentration 10 μg/ml) for 30 minutes at 37°C. Sample was diluted with 2 volumes of TE buffer and added 2.5 M (final concentration) Ammonium acetate (pH 7.7) to precipitate cellular and histone proteins bound to the DNA. DNA was precipitated by adding 2.5 volumes of pre-chilled 95% ethanol. Precipitated DNA was again spooled out with hooked pasture pipette, air dried and finally suspended in 500 μl of TE buffer.

3.1.1.2 Quality check and quantification of isolated DNA

2 μl of DNA sample was diluted with 7 μl of TE buffer and 1 μl of 6X loading buffer and mixed properly. 10 μl of the mixture was loaded in a well of 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide. λ DNA double digest with EcoRI and HindIII (GeNei™, Bangalore, India) served as a molecular weight marker. The agarose gel electrophoresis was carried out for 50V for first 15 minutes and 100V for rest of the period. The gel was visualized, documented and photographed in Gel-documentation instrument (UVIdoc, UVItec Limited, Cambridge, United Kingdom., Model: BTS-26M). The intact double stranded DNA forming a thick single band of high molecular weight confirmed the good quality of genomic DNA. Quality of isolated DNA was reconfirmed by measuring the Optical density (O.D) at $A_{260}/A_{280}$ nm. An $A_{260}/A_{280}$ ratio of 1.8 indicates very pure DNA (Judith et al., 2000). O.D values less or more than 1.8 indicates the presence of proteins and RNA respectively (Ausubel, 1994).

The quality of DNA in the extracted sample was measured spectrophotometrically as a function of its optical density. In a quartz cuvette 5 μl of DNA sample was dissolved in 1 ml of TE buffer and OD was measured at 260 nm against a blank (TE buffer without DNA) in Hitachi UV-VIS spectrophotometer.
An $A_{260}$ of 1.0 indicates 50 ng of double stranded DNA per ml (Ausubel, 1994). The DNA concentration was calculated using following formula.

$$\text{Total DNA (\mu g/ml) = \frac{OD_{260\text{ nm}} \times 50 \times \text{dilution factor}}{1000}}$$

### 3.1.1.3 PCR amplification

Amplification of genomic DNA was performed in a Gradient Palm Cycler (Corbett Research, Australia, Model: CGI-96) and the results were analyzed as described by Williams et al. (1990). A total of 16 decamer primers (Operon Tech., Alameda, USA) were used in the present study. Details of RAPD primers are given in Table 3. Each 20 μl of the PCR mixture consists of 50 ng genomic DNA, 1.5 mM MgCl₂, 200 μM dNTP (dATP, dGTP, dTTP, dCTP), 15 pM primer, 0.5 units of *Taq* DNA polymerase (GeNei™, Bangalore, India). Palm Cycler was programmed with initial denaturation temperature of 94 °C for 4 minutes, 40 cycles of 15 seconds denaturation at 94 °C, 15 seconds annealing at 35 °C, 1.15 minutes for extension at 72°C, followed by final extension of 7 minutes at 72 °C to ensure the completeness of the primer extension.

### 3.1.1.4 Agarose Gel Electrophoresis

Amplified products were separated on agarose gel in a submarine gel electrophoresis unit (GeNei™, Bangalore, India). About 100 ml of 1.2% agarose gel was prepared in 1X TBE buffer [54 g Tris base, 27.5 g Boric acid, 20 ml 0.5 M EDTA (pH 8.0)/liter (5X)], boiled and cooled to 60°C and ethidium bromide (0.5 μg/ml) was added. Then, comb was inserted in the gel casting tray kept on a uniform surface.

Gel was carefully poured into UV-transparent gel casting tray sealed both ends with gum tape without allowing air bubble and was allowed to solidify. After solidification, the comb was removed carefully without damaging the semisolid gel. Gum tape was removed and the gel casting tray along with gel was placed in electrophoretic tank and the tank was filled with 1X TBE buffer, till the gel is completely immersed.
Table 3: List of RAPD primers and their sequences used for diversity analysis in chilli

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Primer</th>
<th>Sequence (5' – 3')</th>
<th>Sl. No.</th>
<th>Primer</th>
<th>Sequence (5' – 3')</th>
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</thead>
<tbody>
<tr>
<td>01</td>
<td>OPJ-01</td>
<td>CCCGGCATAA</td>
<td>09</td>
<td>OPA-17</td>
<td>GACCGCTTGT</td>
</tr>
<tr>
<td>02</td>
<td>OPJ-05</td>
<td>CTCCATGGGG</td>
<td>10</td>
<td>OPA-15</td>
<td>TGCCGAGCTA</td>
</tr>
<tr>
<td>03</td>
<td>OPJ-06</td>
<td>TCGTTCGCA</td>
<td>11</td>
<td>OPA-11</td>
<td>CAATCGCCGT</td>
</tr>
<tr>
<td>04</td>
<td>OPC-13</td>
<td>AAGCCTCGTC</td>
<td>12</td>
<td>OPB-11</td>
<td>GTAGACCCGT</td>
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<tr>
<td>05</td>
<td>OPC-15</td>
<td>GACGGATCAG</td>
<td>13</td>
<td>OPI-01</td>
<td>ACCTGGACAC</td>
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<tr>
<td>06</td>
<td>OPC-03</td>
<td>GGGGGTTTTT</td>
<td>14</td>
<td>OPI-02</td>
<td>GGAGGAGAGG</td>
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<tr>
<td>07</td>
<td>OPA-12</td>
<td>TCGGCGATAG</td>
<td>15</td>
<td>OPI-03</td>
<td>CAGAAGCCCA</td>
</tr>
<tr>
<td>08</td>
<td>OPA-07</td>
<td>CCGATATCCC</td>
<td>16</td>
<td>OPI-06</td>
<td>AAGGCGGCAG</td>
</tr>
</tbody>
</table>
Amplified DNA sample was mixed with 3 μl of 6X gel loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 20% glycerol in deionized water) and spun for 2-5 seconds in microfuge. Samples were gently loaded into the wells using a micropipette. λ DNA double digest with EcoRI and HindIII (GeNei™, Bangalore, India) served as a molecular weight marker. The agarose gel electrophoresis was carried out for 50 V for the first 15 minutes and 100 V for rest of the period. Gels were visualized, documented and photographed in Gel-documentation unit as described by Sambrook and Russell (2001). The photographs were enlarged to a suitable size and printed. Only 3 good representive photographs are presented. All the reactions were repeated and only the consistently reproducible bands were taken into consideration for data analysis.

3.1.2 Variability studies in chilli genotypes

Five representative plants were selected randomly, tagged and observations were recorded in plants for different characters as described below. The data recorded from five plants per treatment was averaged for use in statistical analysis. The following characters were studied.

3.1.2.1 Days to 50 per cent flowering (DFF)

The number of days from date of transplantation to flowering in 50 per cent of plants in the plot was recorded.

3.1.2.2 Plant height (PH)

Height of the plant from ground level to tip of plant at harvesting stage was recorded using a meter scale and expressed in centimeters.

3.1.2.3 Number of primary branches per plant (PB)

Number of branches arising from the main stem above the ground level at harvesting stage was recorded and expressed in numbers.

3.1.2.4 Number of secondary branches per plant (SB)

Number of secondary branches arising from the primary branches was recorded at harvesting stage and expressed in number.
3.1.2.5 Fruit length (FL)

Average fruit length of five fruits from five plants was measured from tip to the base and expressed in centimeters.

3.1.2.6 Fruit diameter (FD)

Average diameter of five fruits from five plants was measured at the top shoulder and expressed in centimeters.

3.1.2.7 Number of fruits per plant (FPP)

The total number of fruits per plant at harvest was counted and recorded.

3.1.2.8 Number of seeds per fruit (SNPF)

The number of seeds from five fruits from each plant was counted and averaged.

3.1.2.9 Seed weight per fruit (SWPF)

The weight of seeds of the same fruits used for recording SWPF was recorded in grams and averaged.

3.1.2.10 Dry fruit yield per plant (DFY)

The fruits from all the pickings from five plants were dried to 10 per cent moisture and the weight was recorded in grams and the average dry fruit yield per plant was calculated.

3.1.3 Statistical analysis

3.1.3.1 Mean

On the basis of individual plant observations, the sample mean for each character in all the accession lines was computed as follows.

\[ Y = \frac{1}{n} \sum_{i=1}^{n} Y_i \]
Where,

\[ Y = \text{Sample mean} \]

\[ Y_i = \text{Individual value} \]

\[ n = \text{Number of observations} \]

### 3.1.3.2 Range

The minimum and maximum value on the basis of individual plant observations was used to indicate the range for a given character.

### 3.1.3.3 Variance

In all the populations, variance was computed for all the characters as follows.

\[
\text{Variance} = \frac{1}{n-1} \left( \sum (Y_i - \overline{Y})^2 \right)
\]

Where,

\[ Y_i = \text{Individual value} \]

\[ \overline{Y} = \text{Population mean} \]

\[ n = \text{Number of observations} \]

Standard deviation (SD) \[ = \sqrt{\text{Variance}} \]

\[ = \frac{\sqrt{\sum d^2}}{n} \]

Where,

\[ d = \text{Deviation of individual value from population mean} \]

\[ n = \text{Number of observations} \]
### 3.1.3.4 Analysis of variance (ANOVA)

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>M.S.S.</th>
<th>Expected values of M.S.S.</th>
<th>Calculated value (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>r-1</td>
<td>MSSr (M_1)</td>
<td>(\sigma^2_e + \sigma^2_g)</td>
<td>(M_1/M_3)</td>
</tr>
<tr>
<td>Genotypes</td>
<td>g-1</td>
<td>MSSg (M_2)</td>
<td>(\sigma^2_e)</td>
<td>(M_2/M_3)</td>
</tr>
<tr>
<td>Error</td>
<td>(r-1) (g-1)</td>
<td>MSSe (M_3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>(rg-1)</td>
<td>(M_1 + M_2 M_3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where,

- \(r\) = Number of replications
- \(g\) = Number of accessions
- \(\text{MSSr}\) = Mean sum of squares due to replication
- \(\text{MSSg}\) = Mean sum of squares due to genotypes
- \(\text{MSSe}\) = Mean sum of squares due to error
- \(\sigma^2_e\) = Error variance
- \(\sigma^2_g\) = Genotypic variance

### 3.1.4 Estimation of genetic parameters

#### 3.1.4.1 Genotypic and phenotypic variances

Genotypic and phenotypic variances were computed based on the expected mean sum of squares from the ANOVA table as given below.

\[ V_g = \frac{M_2 - M_3}{r} \]

\[ V_p = V_g + \text{Ve} \text{ or } \frac{M_2 - M_3}{r} + M_3 \]
Where,

\[ V_g = \text{Genotypic variance} \]
\[ V_p = \text{Phenotypic variance} \]
\[ V_e = \text{Environmental variance} \]

### 3.1.4.2 Phenotypic (PCV) and genotypic (GCV) coefficients of variations

Phenotypic and genotypic coefficients of variations were worked out as suggested by Burton (1953).

\[
\text{PCV} (%) = \frac{\sqrt{\text{Phenotypic variance}}}{\text{General mean of characters}} \times 100
\]

\[
\text{GCV} (%) = \frac{\sqrt{\text{Genotypic variance}}}{\text{General mean of characters}} \times 100
\]

PCV and GCV were classified as suggested by Shivasubramanian and Menon (1973) as follows.

- 0 - 10% - Low
- 10-20 - Moderate
- 20% and above - High

### 3.1.4.3 Heritability

The broad sense heritability \( h^2_{bs} \) was estimated by following the procedure suggested by Weber and Moorthy (1952) as indicated below.

\[
\text{Heritability} = \frac{V_g}{V_p} \times 100
\]

Where, \( V_p \) is the phenotypic variance and \( V_g \) is the genotypic variance of respective accessions.
Heritability percentage was categorized as demonstrated by Robinson et al. (1949).

- **0-30%** - Low
- **30-60%** - Moderate
- **60% and above** - High

### 3.1.4.4 Genetic advance (GA)

It was predicted by using the formula provided by Johnson et al. (1955).

\[
GA = h^2 (bs) \times \sigma_p \times K
\]

Where,

- \( h^2 (bs) \) = Heritability in broad sense
- \( \sigma_p \) = Phenotypic standard deviation of the trait
- \( K \) = Standard selection differential which is 2.06 at 5 per cent selection intensity

### 3.1.4.5 Genetic advance as per cent mean (GA)

It was computed by the formula given below.

\[
GA (%) = \frac{\text{Genetic advance}}{\text{General mean of the character}} \times 100
\]

The genetic advance as per cent mean was categorized as suggested by Johnson et al. (1955).

- **0-10%** - Low
- **10-20%** - Moderate
- **20% and above** - High
Experiment-2: Inheritance studies of pigmentation and morphological characters

3.2 Experimental materials used and methods followed

The present investigation was undertaken during the period 2007-2009. Seeds were sown and grown in the experimental Garden of the Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad, Karnataka, India. The experiment site had medium red sandy loam soil.

3.2.1 Experimental material

Four chilli genotypes namely (Nooji, Phule Jyoti, Yellow Capsicum and Red Capsicum) were used as source material for inheritance studies.

3.2.2 Methods

3.2.2.1 Nursery raising

Nursery beds of 7.2 m length, 1.2 m breadth and 0.15 m height were prepared after mixing recommended dosage of FYM (2 kg/m²) and sterilized by drenching with two percent Capton. Each bed was applied with 500 grams of complex (15:15:15 NPK) fertilizer and mixed thoroughly (Fig.1a). The seeds of all the entries were sown in rows spaced at 10 cm apart. Regular watering and plant protection measures were undertaken. Five week old healthy seedlings were transplanted in a crossing block with the spacing of 60 cm x 60 cm. The plants in the crossing block were nourished with 150:75:75 kgs of NPK per hectare. All other recommended cultivation practices were followed (Anon., 2005). Five weeks old seedlings were transplanted to the main field (Fig.1b). A row to row and plant to plant spacing of 60 cm was followed and the crop was raised as per the recommended package of practices (Anon, 2005).

3.2.2.2 Techniques of hybridization and raising of F₁ and F₂ generations

Hybridization between Nooji (female) and Yellow capsicum (male), Phule Jyoti (female) and Yellow capsicum (male), Nooji (female) and Red capsicum (male), Phule Jyoti (female) and Red capsicum (male) were taken up in the Kharif summer of 2007.
The forceps of the split method proposed by Bhinde (1925) was utilized for crossing the two parents. A young flower from female parents was chosen for crossing. Anthesis commences from about 7.30 am and flowers were emasculated in the evening between 5 pm to 6 pm by opening the petals gently by means of pointed pair of forceps. After the removal of anthers each flower was examined under magnifying lens to ascertain that all the six anthers were removed and stigma was not contaminated by the pollen.

Emasculated flowers were protected with a paper bag. Pollination was carried out between 7.30 am to 8.30 am with the pollen grains collected from freshly opened flowers of desired male parent and gently applied to the stigma of the emasculated female flower. Crossed flowers were covered with paper bags to avoid contamination and tagged with details of female and male parent of the cross and date of pollination. The crossed flowers were protected with butter paper bags until ready for harvest.

Crossed fruits were harvested at red ripe stage. After harvesting, fruits were allowed to dry fully by keeping the fruits for two or three days in the ambient conditions. The seeds were hand extracted from fully riped fruits and dried under room temperature and preserved in butter paper bags labeled with details of entry.

The seeds obtained from crossing were sown along with the thirty five days old seedlings were transplanted into a field. Ridges and furrows were formed 60 cm apart keeping a single plant at each hill. The observations regarding presence or absence of pigmentation in various parts of the plant and morphological and other characters were recorded (Table 4-7).

The flowers of the true F₁ plants were bagged to prevent contamination of foreign pollen and also to get selfed seeds. Hybridization between the parents was continued to get crossed seeds for growing along with F₂.

The F₂ progeny along with F₁ and parents were grown for comparison during Kharif 2009. A total of 1098 F₂ plants were survived up to maturity in the cross between Nooji x Yellow Capsicum. In the cross between Jyoti x Yellow Capsicum, 684 F₂ plants survived. A total of 687 F₂ plants reached maturity in the cross between Nooji x Red capsicum. In the cross between Jyoti x Red Capsicum, a total of 684 F₂ plants could be studied for all the characters.
Table 4: Different traits expressed in Parents and F₁ hybrids of the cross Nooji x Yellow capsicum

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Characters</th>
<th>Parents</th>
<th>F₁ Hybrids</th>
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</thead>
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<td></td>
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<td>Nooji</td>
<td>Yellow Capsicum</td>
</tr>
<tr>
<td>1</td>
<td>Internode colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>2</td>
<td>Node colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>3</td>
<td>Leaf Laminacolour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>4</td>
<td>Petiole colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>5</td>
<td>Petal colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>6</td>
<td>Filament colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>7</td>
<td>Stigma colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>8</td>
<td>Fruit colour</td>
<td>purple</td>
<td>yellow</td>
</tr>
<tr>
<td>9</td>
<td>Calyx</td>
<td>enclosing</td>
<td>not enclosing</td>
</tr>
<tr>
<td>10</td>
<td>Fruit tip</td>
<td>round</td>
<td>lobed</td>
</tr>
<tr>
<td>11</td>
<td>Fruit orientation</td>
<td>up</td>
<td>bend</td>
</tr>
</tbody>
</table>

Pigmentation characters

Morphological and other characters
Table 5: Different characters studied in parents and $F_1$ of the cross Phule Jyothi x Yellow capsicum

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Characters</th>
<th>Parents</th>
<th>$F_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Jyothi</td>
<td>Yellow capsicum</td>
</tr>
<tr>
<td>1.</td>
<td>Stem branching</td>
<td>Solitary</td>
<td>Cluster</td>
</tr>
<tr>
<td>2.</td>
<td>Fruit tip</td>
<td>Pointed</td>
<td>Lobed</td>
</tr>
<tr>
<td>3.</td>
<td>Fruit colour</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>4.</td>
<td>Fruit nature</td>
<td>Solitary</td>
<td>Cluster</td>
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</table>
Table 6: Different traits expressed in parents and $F_1$ generation of the cross Nooji x Red capsicum

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Characters</th>
<th>Parent</th>
<th>$F_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nooji</td>
<td>Red capsicum</td>
</tr>
<tr>
<td>Pigmentation characters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Internode colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>2</td>
<td>Node colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>3</td>
<td>Leaf Lamina colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>4</td>
<td>Petiole colour</td>
<td>purple</td>
<td>green</td>
</tr>
<tr>
<td>5</td>
<td>Petal colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>6</td>
<td>Filament colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>7</td>
<td>Stigma colour</td>
<td>purple</td>
<td>white</td>
</tr>
<tr>
<td>8</td>
<td>Fruit colour</td>
<td>purple</td>
<td>Red</td>
</tr>
<tr>
<td>Morphological and other characters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Calyx</td>
<td>enclosing</td>
<td>not enclosing</td>
</tr>
<tr>
<td>10</td>
<td>Fruit tip</td>
<td>round</td>
<td>lobed</td>
</tr>
<tr>
<td>11</td>
<td>Fruit orientation</td>
<td>up</td>
<td>bend</td>
</tr>
</tbody>
</table>

50
Table 7: Different Characters of parents and F₁ Hybrids generation of the cross Phule Jyothi x Red capsicum

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Characters</th>
<th>Parents</th>
<th></th>
<th>F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Jyothi</td>
<td>Red capsicum</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Stem branching</td>
<td>Solitary</td>
<td>Cluster</td>
<td>Solitary</td>
</tr>
<tr>
<td>2.</td>
<td>Fruit tip</td>
<td>Pointed</td>
<td>Lobed</td>
<td>Pointed</td>
</tr>
<tr>
<td>3.</td>
<td>Fruit nature</td>
<td>Solitary</td>
<td>Cluster</td>
<td>Solitary</td>
</tr>
</tbody>
</table>
The pigmentation and other morphological features of parents, $F_1$ and $F_2$ plants photographed at different stages during the course of this investigation and are presented in Fig. 9 to 16.

3.2.3 **Characters studied for the cross Nooji X Yellow Capsicum**

The characters studied were classified into two groups *viz.*, (a) Characters of anthocyanin pigmentation in different plant parts and (b) Other morphological characters and (c) Other characters were studied for their mode of inheritance.

Observations regarding the presence or absence of pigmentation in various parts of the plant and morphological and other characters were recorded at suitable stages. Magnifying lens of 10 X was used to observe the pigmented character wherever differences between coloured and colourless conditions were not clearly differentiated. Laboratory observations on pigmentation of pericarp were recorded only after complete drying of the fruits.

3.2.3.1 **Anthocyanin pigmentation in Inter node**

This is the smooth solid (when young) or hard (when mature) part of the stem, between the successive nodes. The pigmentation in this part varies from green, faint purple lines to full purple. The observations were taken when the plant was in flowering stage.

3.2.3.2 **Anthocyanin pigmentation in Node**

This is the solid portion of the stem and is the point from where a leaf originates. Its pigmentation varied from light purple, violet to dark purple.

3.2.3.3 **Anthocyanin pigmentation in Leaf blade**

In leaf blade, pigment may be present on lamina, leaf tip, leaf margin and midrib. The colour of leaf blade is either full purple or purple wash or normal green. The pigmentation in the midrib is clearly visible more on the underside of the leaf blade.
3.2.4.4 Stem Branching

Chilli is highly branched and it is much influenced by the genotype. High branching is observed in Byadgi cultivars than sweet pepper. High branching is preferred to prevent fruits coming in contact with soil surface and for easy cultural operations.

3.2.4.5 Type of flower

Flower is bracteolate, pedicellate, bisexual and hypogynous. Flowers are solitary, axillary, sometimes in cluster.

3.2.4.6 Nature of Calyx

Calyx is campanulate, sepals usually five, gamosepalous, persistent and is much shorter than the fruit. Calyx may enclose the fruit base or free.

3.2.4.7 Pigmentation in androecium

Androecium consists of five stamens which are epipetalous. Anthers are purple to white in colour, di-thecous, introse. Anthers dehisce longitudinally by lateral sutures.

3.2.4.8 Gynoecium

Gynoecium consists of 2-3 carpels, superior with numerous ovules in each locule on axile placentation. Style is terminal and linear, stigma is purple to white in colour.

3.2.4.9 Pigmentation in Fruit pericarp

Pericarp is the wall of ripened ovary consisting of layer of cells, which forms a protective layer around the seed. It is differentiated into three layers as epicarp, mesocarp and endocarp. The colour of pericarp varies from purple to different shades of red or yellow.

3.2.5 Other characters

3.2.5.1 Fruit orientation

If the fruit is upright it will be considered erect, while if the fruit is bent at 45 degree it is considered as pendent.
3.2.5.2 Fruit nature

Fruits may be solitary or clustered.

3.2.6 Statistical analysis of genetic characters

The expected values corresponding to the observed value for each character were calculated based on the ratio hypothesis. The chi-square test was applied to test the significance of deviation using the formula.

\[
X^2 = \sum \frac{(a - t)^2}{t} \quad \text{with (n-1) df}
\]

Where,

\[\sum\] = Summation over all classes

\[a\] = Observed frequencies

\[t\] = Expected frequencies and

\[n\] = Number of classes

The genetic hypothesis was confirmed fit when the calculated chi-square value was less than the table value at 5 per cent level at respective degrees of freedom. Apart from fitting the genetic ratios, the chi-square was also used for testing the genetic association of the genes controlling the characters in joint segregation. In case of two gene interactions of the four phenotypic classes of joint segregation, the class consisting dominant phenotypes for both the characters was designated as AB (doubly dominant), dominant phenotype for the first character alone as A (single dominant), dominant phenotype for the second character alone as B (singly dominant) and the recessive phenotype for both the characters, as ab (doubly recessive). The expected values corresponding to the observed values of these classes were calculated based on the joint segregation ratio. Chi-square test was applied to test the significance of deviations. The characters under study were considered as independent of each other when the chi-square value calculated was less than or equal to the table value at 5 per cent level at
respective degrees of freedom. On the other hand, when the chi-square value was more than the table value, the data was tested for Pleiotropy or linkage or pleiotrophy cum linkage. Pleiotropy was shown in cases wherever there was absence of one or both recombinant classes and in some cases even when cross overs are present. In testing for linkage, the total chi-square was further partitioned into (1) one degree of freedom to test the ratio of first character, (2) one degree of freedom to test the ratio of second character and (3) the remaining one degree of freedom to test the genetic association of both the characters (Richharia et al., 1966). The sum of the two chi-square values of two individual ratios was subtracted from the total chi-square value to get the chi-square value for joint segregation with one degree of freedom. When this value was more than the table value (3.841) at 5 per cent level for one degree of freedom, the characters were taken as linked.

The recombination values were estimated by adopting the Product Ratio (PR) method proposed by Fischer and Balamakund (1928). The formula of ‘P’ in terms of product ratio ‘R’ expressed in quadratic form developed by Kolhe (1972) was used.

Formulae of expected frequencies of four phenotypic classes based on linkage or pleiotropy cum linkage were derived by following the method of Bhat (1950) for their utilization in computing linkage values by the two methods mentioned earlier. Various formulae of expected frequencies of four phenotypic classes developed and used for estimating linkage values in the present investigation are presented in table 10 to 48.

The recombination values were arranged pair wise and the linear order of the loci was determined in the trial construction of linkage maps. The map distances were corrected by using the formula developed by Kosambi (1944), as given below:

\[
Y_{12} = \frac{Y_1 + Y_2}{1 + 4Y_1Y_2}
\]

Where, \(Y_1\) and \(Y_2\) are the recombination values of adjacent segments and \(Y_{12}\) is the recombination value of larger section comprising both the segments.
Experiment-3: Histological basis of resistance to thrips and mites

3.3 Material

Two varieties VN2 and Byadgi kaddi which are resistant and susceptible respectively to thrips and mites were used for the study.

3.3.1 Collection of samples for anatomical and histological studies

3.3.1.1 Leaves

Fifth leaf from the top was collected from five plants random and pooled together and were analyzed for anatomical and histological observations (Johanson, 1940 and Jensen, 1962).

3.3.1.2 Stem

The stem tip was collected from the growing point of by cutting 2-3 cms young shoot portion. Few plants were selected from each plot and samples were pooled together and analyzed for anatomical and histological characters.

3.3.1.3 Squares

The squares were collected randomly from 5 plants and pooled together for anatomical and histological observations.

3.3.1.4 Petioles

The sampling of petiole was done from the same leaf collected from the stem analyzed for anatomical and histological characters.

3.3.2 Micro technique procedures for Anatomical and Histological studies

3.3.2.1 Fixation

Both resistant and susceptible stem, petiole and leaf samples were collected separately and fixed in formalene acetic acid and 70 per cent alcohol (FAA) in the ratio of 1:1:18, the material was allowed to remain in the solution for 24 hours.
3.3.2.2 Dehydration

Fixed material was thoroughly washed in 70 per cent alcohol and further dehydrated by passing through 80 per cent, 90 per cent and absolute alcohol. The dehydration was carried out at least for two hours interval. Further dehydration was continued using alcohol and paraffin solvent butanol series.

3.3.3 Details of the alcohol, butanol grades are given below

3.3.3.1 Paraffin infiltration

For the process of infiltration, paraffin with a melting point of 58 to 68°C was selected. Small quantity of paraffin was added to specimen tubes containing dehydrated plant material for cold infiltration. Further specimen tubes were kept in hot air oven maintained at 50°C above melting point. Fresh molten paraffin was added at an interval of four hours till last traces of butanol were removed.

3.3.3.2 Embedding

After the removal of n-butanol from the dehydrated material, it was embedded in paraffin wax by adopting paper boat technique. The paper boats of appropriate size were prepared and inner surface of paper boat was smeared with glycerin. The dehydrated plant material with molten wax was poured into the boats immediately followed by re-boiled molten wax. For the easy cutting of blocks the material was arranged in proper way in linear rows.

3.3.3.3 Microtomy

From the paraffin blocks containing leaf, petiole and stem, thin sections of 7μm were obtained using rotary microtome.

3.3.3.4 Affixing the sections

Gelatin (one per cent) was used as an adhesive to fix the sections to slides, which was prepared in warm, distilled water. To this small quantity of potassium dichromate crystals were added and later it was filtered and used to fix the section to slides.
Small amount of gelatin was smeared on the down sides and ribbons of convenient size were spread carefully. Further the sides were warmed and hot plate maintained at 45°C for further stretching of the sections. The sides were tilted to remove excess gelatin, later the sections were dried at room temperature for 72 hours and stored in a clean slide box.

3.3.4 Staining procedure for anatomical studies

The sections were stained by using a combination of stains for anatomical observations and for photography of tissue sections.

3.3.4.1 Deparaffinizing and hydrating the sections

Sections were deparaffinized by using xylene and later hydrated using the alcohol series as per the requirement. Hydration of the sections was carried out following the steps listed below.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Duration of the treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene + alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>90 per cent alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>70 per cent alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>50 per cent alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Water</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

3.3.4.2 Preparation of the safranin stain

A stock solution of safranin was made by dissolving 1 g of safranin in 100 ml of 50 per cent alcohol.

3.3.4.3 Preparation of fast green stain

Fast green stain was prepared by adding 0.5 g of fast green in a mixture of 100 ml of 95 per cent alcohol.
3.3.4.4 Preparation of clove oil mixtures

Clove oil mixture was prepared by mixing 50 per cent clove oil with 50 per cent xylene.

3.3.4.5 Staining Schedule for histological studies (Safranin and Fast green method)

1. The sections were deparaffinized (5 minutes) as described earlier.
2. Stained in safranin for 24 hours.
3. Excess stain was washed in running water.
4. Gradually dehydrated by passing rapidly in a series of alcohol like 50, 70, 90 per cent and absolute alcohol.
5. Counter stained with fast green for a short period of 3 minutes.
6. Then passed in 90 per cent alcohol and absolute alcohol.
7. Excess stain was cleared by clove oil mixture.
8. Passed in xylene and mounted in DPX.
9. Various anatomical observations were recorded at 10x magnification

3.3.5 Micrometry

Observations on the number of cells per unit area, thickness of the material and size of different tissues of the plant samples were recorded by using calibrated ocular micrometer standardized with the help of stage micrometer.

3.3.6 Photomicrography

The histochemically stained sections were photographed using ‘Axiostar plus’ (Carl Zeiss) Bright field / Fluorescent Modular microscope and Cannon’s Power Shot G2 digital camera with higher resolution.

The fluorescent microscopic observations were made at University Scientific Instrumentation Center, KUD, Dharwad.
Experiment-4: Histological studies in male sterile and male fertile VN2 lines

3.4 Experimental details

Male sterile VN2 lines and Male fertile VN2 lines maintained at UAS Dharwad were used as source material for Male sterility studies.

Comparative histological studies of anther and pollen grains from the male fertile and sterile plants were studied using light microscope by adopting standard method of microtomy (Johanson, 1940 and Jensen, 1962).

3.4.1 Sampling

The flower buds of different size were sampled by selecting important representative developmental stages to study anther development. They were categorized into different stages based on the size of the bud. While collecting the buds care was taken to see that the sample included all the important successive stages of anther development.

3.4.2 Fixation

Flower buds of different sizes were fixed in the standard fixative, carnoys-A (3 parts of absolute alcohol and one part of glacial acetic acid) prepared fresh. The flower buds were allowed to remain in the fixative for 48 hour. Afterwards, flower buds were transferred to 70 % alcohol.

The process of dehydration, paraffin infiltration, embedding, micrometry, affixing the sections, deparaffinization of the sections and photomicrography was done following the procedures given in the section 3.3.2.2, 3.3.3.1, 3.3.3.2, 3.3.3.3, 3.3.3.4, 3.3.4.1 and 3.3.6.
3.4.3 Histochemical staining

3.4.3.1 Total proteins: Amido black 10B method (O'Brien and McCully, 1981).

Staining procedure

i. Hydrated sections were incubated for 15 minutes in 0.05% amido black 10B at room temperature.

ii. Sections were rinsed in 7% acetic acid for 1 minute, air dried, cleared in xylol and mounted using DPX.