3. MATERIALS AND METHODS

3.1 MATERIALS

*Commiphora wightii* is a small tree reaching up to 4m. It is branched and thorny (Figure 3.1A & B). Leaves are small ranging 1 to 5 cm in length and 0.5 to 2.5 cm breadth, serrate in margin and ovate in shape. Flowers are red or pink in colour (Figure 3.1D). Fruits are pulpy and are ovate in shape (Figure 3.1E). This plant is found in the areas having harsh and rough climates. It is more commonly seen in rocky mountains and unfertile soils belonging to arid and semi arid zones of Rajasthan, Gujarat and Madhya Pradesh. Natural regeneration is very poor in guggul. It can be propagated by seeds and also vegetatively. It is listed in Red Data List of IUCN as threatened plant and now it is becoming endangered. It produces guggul gum (Figure 3.1C) which is a very important constituent of many useful drugs. Depletion in its population has been observed in its natural habitats, primarily due to over-exploitation, unsustainable and destructive methods of gum-extraction coupled with slow growth and poor regeneration of the plant (Jain and Nadgauda 2013). Present population is represented by female plants, male and andromonoecious plants are extremely rare.

Present studies were carried out with two male plants and three female plants growing at AFRI experimental area. These five plants were used to compare male and female plants as per our objectives. Other studies with progenies (219) of 30 mother plants (females) were raised in mist poly house through seed and stem cuttings as described in following methods.

3.2 METHODS

3.2.1 FIELD WORK

3.2.1.1 Survey and selection of site

Survey was conducted in year 2011-12 to identify six different sites, three each from Rajasthan and Gujarat state. The major criterion of site selection was the availability of good population density and proper protection. The sites having natural population of guggul were selected. The GPS locations and detailed information of all these six sites are given in table 3.1.
### Table 3.1: Brief information about all six sites.

<table>
<thead>
<tr>
<th>SITES</th>
<th>RAJASTHAN</th>
<th>GUJARAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Kayalana, Jodhpur</td>
<td>Nardas ka gurha, Rajsamand</td>
</tr>
<tr>
<td>GPS Location</td>
<td>26°20 N, 73°15 E</td>
<td>24°46 N, 73°28 E</td>
</tr>
<tr>
<td>Total no. of plants selected</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

### 3.2.1.2 Identification of mother plants:

Five healthy plants from each field were selected as mother plants for raising progenies and mother stock plants for isozymes and DNA markers studies. Their flowers were observed to know the sex of the plant. Mature seeds and stem cuttings (10 nos. from each plant) from all those selected healthy plants were then collected for establishing them at AFRI mist polyhouse.

### 3.2.1.3 Identification of male/andromonoecious plants

All the six sites as given in table (3.1) were surveyed thoroughly but not even a single male or andromonoecious plants were identified at these sites. Finally two male plants were available in AFRI germplasm collected by Dr. D.K. Mishra, along with three female plants raised at the same time having different collection locations were used to study the differences on morphological and chemical parameters/characters.

### 3.2.1.4 Collection of stem cuttings and seeds

Collection of mature guggul fruits from each selected mother plant was done during February to May month of year 2011. The fruits were collected in poly bags and tagged with identity. Cuttings of all mother plants (30-40 cm) were also collected and grown in AFRI mist poly house (Figure 3.2C).

### 3.2.1.5 Establishment of mother clones and their progenies

The seeds were obtained after removing epicarp and mesocarp of fruits. Seeds so obtained were then air dried completely. Seeds were kept for germination
in the poly house of AFRI. White coloured seeds (Figure 3.2A) were non viable while black-coloured viable seeds (Figure 3.2B) germinated within 5-16 days after sowing. Seeds of three plants from Kachchh region germinated in good number but subsequent survival was very poor. Total 8 progenies from each mother plant were selected randomly except 3 plants of Kachchh region (only 1 progeny from each). Finally total 219 plants belonging to 30 different mother trees were established through seeds (Figure 3.2D).

3.2.2 DIFFERENCES BETWEEN MALE AND FEMALE PLANTS

Differences between male and female *Commiphora wightii* on the basis of morphological markers and Z- guggulsterone content were observed. The studies were carried out on 5 genotypes including two male M1 and M2 and three females F1, F2 and F3 genotypes of *Commiphora wightii*.

3.2.2.1 Morphological differences

Morphological studies were carried out with a view to identify some strong morphological markers to distinguish male and female plants even during non flowering period also. All the genotypes were propagated by cutting and the observations were noted for the same age group and at the same time in the mid of June month, 2013. Sixteen different morphological parameters were observed out of which eight were qualitative and eight were quantitative.

3.2.2.1.1 Growth Characteristics

Growth parameters of plant like plant shape, height, canopy, growth habit, and crown size were observed.

I) Plant shape- The overall shape of the plant was evaluated visually on the basis of standard terminology used in taxonomy.

II) Growth habit- The growth of the plant was evaluated on visual assessment basis.

III) Canopy- The canopy of the plant was also observed on the basis of visual evaluation among all the genotypes.
IV) Plant height- Plant height was measured in cm from ground adjacent to the stem to the tip of the highest branch of plant using graduated pole.

V) Crown- Crown diameter was measured in cm of width of the branches at their greatest extent at two right angle directions using measurement tape. The average of the readings was considered as crown diameter.

3.2.2.1.2 Branching pattern

Branching parameters like branching type, number of branches and branching angles were measured to know the exact branching pattern. In addition differences in stem bark colour of plant were also observed.

I) Branching type- The type of branching was observed on the basis of visual assessment.

II) Number of branches- The numbers of branches were counted at three different levels viz. primary, secondary and tertiary starting from the base of each genotype.

III) Branching angle- The angle of branching was evaluated using divider and compass at three different orders. I order is the branching angle between primary and secondary branches, II order is the branching angle between secondary and tertiary branches and III order is the branching angle between tertiary and quaternary branches.

IV) Stem bark colour- The stem bark was taken from the stem of same diameter from same location of every plant and then assessed visually.

3.2.2.1.3 Leaf morphology

Different leaf parameters like leaf shape, leaf margin, leaf size, number of leaves, leaf colour, leaf area and leaf stomata were observed.

I) Leaf shape- Ten fully matured leaves from each genotype were taken and assessed visually to evaluate leaf shape.

II) Leaf margins- To compare leaf margins ten fully expanded matured leaves from each genotype were taken and assessed visually.
III) **Leaf size**- Leaf length of ten fully mature leaves was measured using measurement scale from each genotype.

IV) **Number of leaves**- Three apical twigs (15 cm long) were collected from each plant and leaves on each twig were counted excluding the small leaves at the top of the twig.

V) **Leaf area**- Three fully mature leaves from each plant were taken to calculate leaf area using graph paper (Green-armytage 2008). The leaf was placed on graph paper having 1 cm² square grid boxes and then boundary of leaf was drawn. Leaf area was calculated by using following formula:

\[
\text{Leaf area} = \text{No. of grids completely covered} + \frac{\text{no. of grids incompletely covered}}{2}
\]

VI) **Leaf stomata**- Fresh mature leaves of all the genotypes were collected to observe the no. of stomata. Leaves were washed and dried before removing epidermal layers.

To peel the epidermal layer first of all a small piece of transparent cello tape was taken, folded from one end (to make it easy to peel off again) and placed on the bottom side (lower epidermal layer) of the leaf. A thin coat of clear nail polish was then painted half on tape and half on leaf surface and when the nail polish was completely dried the tape was peeled off carefully with dried nail polish stuck to it. Next, this dried layer of nail polish was carefully placed on microscope to observe the no. of stomata (Grant and Vatnick 2004). Same procedure was followed for upper epidermal layer. Counting of stomata was performed on both adaxial and abaxial epidermal layer of each genotype in 3 different microscopic fields of microscope. An average no. of stomata and epidermal cells per microscopic field (400X magnification) were calculated and stomatal index was calculated using the equation of Salisbury (1927).

\[
\text{Stomatal index} = \frac{\text{no. of stomata} \times 100}{\text{no. of stomata} + \text{no. of epidermal cells}}
\]
Stomatal density was also determined by calculating stomatas per mm\(^2\). For this number of stomatas present in a particular area of a grid was calculated using motic image analyser and then it is divided by that area of grid (Grant et al. 2006).

\[
\text{Stomatal density} = \frac{\text{no of stomata in particular grid}}{\text{Area of that grid}}
\]

### 3.2.2.2 Difference in Z- Guggulsterone

A non destructive method (Agarwal et al. 2000) i.e. isolation of guggulsterone from aerial branches was used in present studies. The comparison of amount of guggulsterone present in male (2) and female (3) plant samples of Rajasthan was done. This investigation was performed in the laboratory of Directorate of Medicinal Aromatic Plants Research, Anand.

The aerial branches from selected male and female plants were collected and dried under shade and powdered in mixer grinder. 50 gm powder from each sample were taken and dissolved in 300 ml ethylacetate. It was extracted on water bath for \(\frac{1}{2}\) hour and then filtered. To the residue 200 ml ethylacetate was added and extracted again on water bath. The procedure was repeated twice. The extract was then filtered and the filtrate was evaporated on rotary evaporator to get a green coloured thick viscous extract. This green coloured thick viscous extract was dissolved in 100 ml methanol (HPLC grade) and 2 ml of this methanol extract was then transferred to vial and centrifuged for two times on 5000 rpm for 5 minutes. Supernatant was directly used as sample for HPLC (Perkin Elmer, USA) analysis. 10 µl of sample was loaded in HPLC. High performance liquid chromatography was performed at 242 nm wavelength using standard reference (M/s Natural Remedies, Bangalore, India). The mobile phase used was 0.1% acetonitrile and formic acid/H\(_2\)O (60:40) and flow rate adjusted at 1.0 ml/min. The column was used of RP-18 (250mmX4.6mm.5µm, Merck).

The peak of standard Z- guggulsterone was observed (Graph 3.1) and then readings of the samples were compared to know the exact amount present. The data was then analysed and percentage of Z- guggulsterone was calculated.
Graph 3.1: Chromatogram showing peak of Z- guggulsterone.

3.2.3 MOTHER PLANT AND PROGENY STUDIES

3.2.3.1 Biochemical marker (Isozyme) study:

To assess the breeding behavior in *Commiphora wightii*, mother plants and their progenies were studied using isozymes as genetic markers. Three isozyme namely catalase, peroxidase and acid phosphatase were selected on the basis of previous literature. All three isozymes studies were performed using Native PAGE method (Davis 1964). Out of three isozymes, two isozyme namely peroxidase (E.C. no. 1.11.1.7) and acid phosphatase (E.C. no. 3.1.3.2) has been studied in depth whereas third isozyme namely catalase (E.C. no. 1.11.1.6) was found below detectable limits so it was excluded in present studies.

3.2.3.1.1 Stock solutions

All the stock solutions used for protein extraction, polyacrylamide gel electrophoresis and for staining of isozymes were prepared as described in table 3.2.
**Table 3.2: Stock solutions preparation for isozyme studies.**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Name of the reagent</th>
<th>Method of preparation of stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30% acrylamide/bisacrylamide solution.</td>
<td>29.2 gm acrylamide (Himedia) and 0.8 gm bisacrylamide (Himedia) were weighed and dissolved in 100 ml d.w. and stored at 4°C in dark.</td>
</tr>
<tr>
<td>B</td>
<td>Separating/resolving gel buffer</td>
<td>18.15 gm tris was weighed and dissolved in 100 ml distilled water. 8.8 pH was maintained and stored at 4°C.</td>
</tr>
<tr>
<td>C</td>
<td>Stacking gel buffer.</td>
<td>6 gm tris (Merck) was dissolved in 100 ml of distilled water. pH 6.8 was maintained and stored at 4°C.</td>
</tr>
<tr>
<td>D</td>
<td>Sample loading buffer</td>
<td>For making 8 ml of sample loading buffer 3.8 ml of distilled water was added to 1 ml of stacking buffer, to which 2.8 ml of glycerol and 0.4 ml of bromophenol was also added. The sample was diluted at least 1:4 with sample loading buffer. Stored at RT (30°C).</td>
</tr>
<tr>
<td>E</td>
<td>Running buffer/Electrode/Tank buffer</td>
<td>15 gm tris and 72 gm glycine (Himedia) were dissolved in 1 l distilled water. Stored at 4°C but maintained at 37°C before use. To make working solution 300 ml of stock solution was then added to 1200 ml distilled water which can be used three times to run the gel.</td>
</tr>
</tbody>
</table>
| F     | Phosphate buffer | MSP solution-3.12 gm monobasic sodium phosphate (NaH₂PO₄·2H₂O, Sd fine) was dissolved in 100 ml d.w. to make 0.2 M solution.  
DSP solution-2.83 gm dibasic sodium phosphate (Na₂HPO₄, Sd fine) in 100 ml d.w. to make 0.2 M solution.  
Phosphate buffer of pH-6 was prepared by mixing 87.7 ml of MSP and 12.3 ml of DSP. |

### 3.2.3.1.2 Preparation of sample from fresh leaves:

For analysis of isozymes, 500 mg leaves from each of plant material were extracted in 5 ml of 0.2M chilled phosphate buffer (Stock F Table 3.2) manually in chilled paste mortar and centrifuged at 10,000 rpm for 30 min at 10°C. The supernatent was treated directly as enzyme extract and was used as such for analytical purpose.
3.2.3.1.3 Preparation and casting of poly acrylamide gel:

Preparation of resolving gel: For preparation of resolving gel of definite pore size stock A + stock B (Table 3.2) and water are mixed in definite volume (as mentioned in the table 3.3) then freshly prepared APS (0.1 gm in 1 ml of distilled water) and TEMED (Merck) solutions were added. Solution was mixed and poured into gel sandwich. Gel was overlaid with water and unit was allowed to stand for 20 minutes for polymerisation.

Preparation of stacking gel: To cast stacking gel stock A+ stock C (Table 3.2) and water were mixed in definite volume (Table 3.3) then freshly prepared APS (as mentioned above) and TEMED solutions were added. The water overby on running gel was discarded. The stacking gel solution was then introduced to fill the gel cast. The comb was inserted immediately taking care not to trap any bubbles. The stacking gel was allowed to polymerise for half an hour.

Table 3.3: Preparation of resolving and stacking gels.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Resolving gel 10 ml</th>
<th>Stacking gel 5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4.06 ml</td>
<td>3.45 ml</td>
</tr>
<tr>
<td>Stock A (30%Acryl/Bisacrylamide)</td>
<td>3.33 ml</td>
<td>0.85 ml</td>
</tr>
<tr>
<td>Stock B (resolving gel buffer)</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Stock C (stacking gel buffer)</td>
<td>-</td>
<td>0.62 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

3.2.3.1.4 Loading and running of sample

The wells so formed after removing combs were thoroughly washed at first with distilled water and then electrophoresis buffer in order to remove any unpolymerised gel. After washing of wells, the apparatus was then set in electrophoresis unit and filled with tank buffer (Stock E Table 3.2). The standards Wheat germ acid phosphatase (Sigma Aldrich)/ Horse radish peroxidase (Sigma
Materials and Methods

Aldrich) and protein samples were then mixed with equal sample loading buffer and 15μl of this mixture was loaded in each well (Stock D Table 3.2).

For running, the assembly was attached to power unit at constant current of 40 mA. While running, the gel was maintained at low temperature (10°C to 11°C) in cold room. After the tracking dye reaches the lower end of the gel indicating the movement of samples, the gel was taken out and washed gently with doubled distilled water.

3.2.3.1.5 Staining of gel and observation of banding patterns.

Peroxidase- For peroxidase (PRX), gel was stained using protocol of Mydlarz and Harvell (2006) with slight modification. The electrophoresed gels were stained with freshly prepared 0.5% ethanolic solution of O-dianisidine (Loba chemie). Then 1% H₂O₂ solution (Sd fine) was added in a dropwise manner till light brown coloured bands appear. The gel was then placed in distilled water overnight for clear resolution of reddish brown coloured bands.

Acid Phosphatase- For acid phosphatase (ACP), gel was stained in a solution of 0.05M acetate buffer of pH 4.8 containing 1mg/ml sodium-1-naphthyl phosphate (Himedia) and 1mg/ml Fast garnet GBC (o-amino azotoluene, Sigma Aldrich ) as a diazonium salt (Ghosh et al. 1983). Staining was performed overnight at 10°C. Next day dark brown coloured bands were produced. The stained gels were then photographed using Nikon camera and were dried using gel drier (Rapiddry), coated with glycerine and stored in zip lock polythenes.

3.2.3.2 Molecular Marker (RAPD) study:

Random amplified polymorphic DNA (RAPD) analysis was also carried out to study the mother plants and their progenies. Standard protocols were used for the isolation of DNA and RAPD analysis (Samantaray et al. 2009). All 6 primers of OPA and OPN series identified as polymorphic and suitable for Commiphora wightii were used.

3.2.3.2.1 Stock solutions: Stock solutions of reagents for DNA isolation, purification, quantification and agarose gel electrophoresis were prepared and stored at room temperature (Table 3.4).
Table 3.4: Preparation of stock solutions for RAPD studies.

<table>
<thead>
<tr>
<th>Sto</th>
<th>Name of reagents</th>
<th>Method for preparation of reagent solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tris HCl (1M)</td>
<td>121.14 gm Tris base (Merck) was dissolved in 900 ml double distilled water and further d.w. was added to make it 1 l. The pH of this solution was 8.0.</td>
</tr>
<tr>
<td>B</td>
<td>Ethylene Diamine Tetra Acetic acid (0.5M EDTA)</td>
<td>186.1 gm of EDTA (Himedia) was dissolved in 800 ml double distilled water and volume was made to 1 l (pH 8.0).</td>
</tr>
<tr>
<td>C</td>
<td>Sodium chloride (NaCl)</td>
<td>Dissolved 292.2 gm of NaCl (Merck) in 800 ml of double distilled water and stirred till NaCl was dissolved completely and made the volume to 1 litre.</td>
</tr>
<tr>
<td>D</td>
<td>CTAB (5%)</td>
<td>5 gm of CTAB (Himedia) was dissolved in 100 ml of double distilled water. It is a strong detergent that helps lyse the cell membrane. CTAB bricks with DNA and form DNA-CTAB complex. Hence, when isopropanol is added only DNA complex gets precipitated.</td>
</tr>
<tr>
<td>E</td>
<td>Ethidium bromide</td>
<td>1 gm ethidium bromide was added in 100 ml of distilled water. It binds to DNA molecules by intercalating between adjacent base pairs and illuminates with UV light.</td>
</tr>
<tr>
<td>F</td>
<td>Loading dye</td>
<td>0.09% bromophenol blue + 60% glycerol + 60 mM EDTA used as a color marker to monitor the process of agarose gel electrophoresis.</td>
</tr>
<tr>
<td>G</td>
<td>TAE buffer (50 X Tris acetic acid EDTA buffer)</td>
<td>242.28 gm 2 M Tris base, 57.1 ml Glacial acetic acid and 100 ml (pH 8.0) 0.5 M Na₂EDTA was taken and made to 1 ltr using D.W.</td>
</tr>
<tr>
<td>H</td>
<td>Preparation of extraction buffer (10 ml)</td>
<td>Extraction buffer for DNA extraction was freshly prepared as per the detail given here: (Tris HCl- 100mM), (EDTA-20 mM), (NaCl-5M), (PVP-2%), (β mercaptoethanol- 0.2%), (CTAB-5%).</td>
</tr>
</tbody>
</table>

3.2.3.2 Isolation of genomic DNA

Total genomic DNA was extracted from selected mother plants and their progenies by the CTAB method (Samantaray et al. 2009). DNA isolation and purification is of crucial importance since pure DNA is an absolute requirement for the DNA fingerprinting.
Fresh juvenile leaves were taken from selected plants grown in AFRI poly house between 10:00 AM to 1:00 PM. Leaves were thoroughly washed, dried, deveined, weighed up to 0.5 gm and then grinded to a fine powder in pestle and mortar using liquid nitrogen. The powder was then suspended in 2ml sterile centrifuge tube containing 1 ml of pre warmed extraction buffer (Stock H Table 3.4), 20 µg PVP (To remove phenolic compound by making hydrogen bonding) and 8.8 µg ascorbic acid. The extraction buffer contains CTAB (Stock D Table 3.4) as detergent to disrupt the membranes, β - mercaptoethanol (A reducing agent to remove tannins and poly phenols) and EDTA (Stock B Table 3.4) as chelating agent which helps to inactivate nuclease that are released from the plant cell and can cause serious problem by degradation of the genomic DNA. After mixing, the tubes were incubated on water bath at 65°C for 1 h 20 min. During incubation gentle inversions of tubes were done at every 10-15 min. After incubation the tubes were then allowed to cool at 30°C then equal volume of chloroform: isoamylalcohol (24:1) was added to each tube. The tubes were inverted gently for 10-15 min to form an emulsion and then centrifuged at 3000 rpm at 20°C for 25 minutes. After centrifugation upper aqueous layer was pipette out carefully into a new tube containing 250 µl of 5M NaCl (Stock C Table 3.4) without disturbing the interphase. Chilled ethanol (100%) was then added to the supernatant to precipitate the DNA. Tubes were centrifuged for 5 min at 13000 rpm at room temperature, to pellet the DNA and the pellets so obtained were then washed thoroughly with 70% alcohol for 2-3 times. The pellets were air dried at Laminar Air Flow bench, dissolved in 200µl of TE buffer (10 mM Tris HCl and 0.5 M EDTA, volume was made to 1 litre using double distilled water) or nuclease free water and stored at 4°C overnight.

3.2.3.2.2 Purification of DNA

Isolated DNA was purified next day by adding 2µl Rnase-A (10mg/ml, Fermentas- DNase and protease free) to the vial and then incubated at 37°C for 1 h to avoid RNA contamination. After incubation equal amount of chloroform: isoamylalcohol :: 4:1 (Merck) treatment was given (for the removal of contaminating molecules) to each vial and mild shaking (by inverting) was done for 10 minutes, results in separation of organic and aqueous phase (DNA). Tubes were then
centrifuged for 20 min at 3000 rpm at 20°C. Supernatant was then collected in a separate sterile vial and the above step was repeated 2-3 times depending upon the impurities present. After centrifugation supernatant was pipette out carefully into a new tube and 1/10 volume of 3M sodium acetate (Merck) was added. Chilled ethanol (100%) was then added to the supernatant to precipitate the DNA. Tubes were centrifuged for 5 min at 13000 rpm, to pellet the DNA. Pellets were then washed thoroughly with 70% alcohol for 2-3 times. The pellets were air dried at LAF bench, dissolved in 50µl of TE buffer or nuclease free water (NFW) and stored at -20°C.

3.2.3.2.3 Quantification of DNA

The genomic DNA isolated was quantified spectrophotometrically by measuring absorbance at 260 nm and purity of DNA was checked using O.D.₂₆₀/O.D.₂₈₀ ratio. Good quality DNA having this ratio between 1.7- 2.0 was selected.

Sterile quartz cuvettes were taken and filled with TE buffer solvent as blank. Zero value was set at 230, 260, 280 and 320 nm in spectrophotometer. Sample DNA was diluted up to 1000µl dilution factor (Dilution factor=3000/3=1000µl). Optical density was then taken at 230, 260, 280 and 320 nm. DNA concentration was calculated using following formula:-

\[
\text{DNA concentration (µg/µl)} = \frac{\text{O.D.}_260 \times 50 \times \text{dilution factor}}{1000}
\]

3.2.3.2.4 Amplification conditions

DNA sample were amplified using thermal cycler machine, method as described by Williams et al. (1990) with certain modifications. Stock DNA was diluted to make a working solution of 30 ng/µl for amplification. Six highly polymorphic random oligonucleotide primers (10 nucleotides base pairs) used in *Commiphora wightii* were selected from the literature for RAPD analysis are given in table 3.5.
Table 3.5: Sequences of six random primers used in RAPD analysis

<table>
<thead>
<tr>
<th>Code</th>
<th>5' to 3' sequence</th>
<th>Molecular weight</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA 04</td>
<td>(5´-AATCGGGCTG -3´)</td>
<td>3068.06</td>
<td>4.2</td>
</tr>
<tr>
<td>OPA 09</td>
<td>(5´-GGGTAACGCC -3´)</td>
<td>3053.06</td>
<td>4.3</td>
</tr>
<tr>
<td>OPA 20</td>
<td>(5´-GTTGCGATCC-3´)</td>
<td>3019.04</td>
<td>4.6</td>
</tr>
<tr>
<td>OPN 06</td>
<td>(5´-GAGAGGCACA-3´)</td>
<td>3046.07</td>
<td>4.4</td>
</tr>
<tr>
<td>OPN 16</td>
<td>(5´-AAGCGACCTG-3´)</td>
<td>3037.06</td>
<td>4.5</td>
</tr>
<tr>
<td>OPN-20</td>
<td>(5´-GGTGCTCCGT-3´)</td>
<td>3035.04</td>
<td>4.6</td>
</tr>
</tbody>
</table>

- **Master mix (24 µl) for amplification reaction**
  
  Amplification reaction mixture was prepared in 200 µl thin walled sterile PCR tubes according to Table 3.6.

Table 3.6: PCR master mix components and volume for each PCR tube.

<table>
<thead>
<tr>
<th>PCR master mix</th>
<th>1X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>16.8 µl</td>
</tr>
<tr>
<td>10X PCR buffer with MgCl₂ (Fermentas)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Primer (Xcelris Genomics)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTP (Fermentas)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (Fermentas)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>24 µl</td>
</tr>
</tbody>
</table>

Except template DNA the master mix was distributed to PCR tubes (24µl/tube) and later 1 µl of template DNA from the respective isolates was added making the final volume of 25µl. The mixture was then given a short spin to mix the contents. The conditions and steps for DNA amplification in thermal cycler are given in table 3.7.
3.2.3.2.5 Separation and detection of amplified products

Amplified PCR products were separated and detected using Agarose gel electrophoresis technique (Sambrook and Russell 2001). For this 1.4 gm of Agarose (Genaxy) was weighed and added to a 250 ml conical flask containing 100 ml of 1 x TAE buffer (Stock G Table 3.4). The agarose was melted by heating the solution on an oven and the solution was stirred to ensure even mixing and complete dissolution of agarose. The solution was then cooled to about 50°C. 3µl of ethidium bromide (Stock E Table 3.4) solution was then added. The solution was mixed and poured into the gel casting platform after inserting the comb in the trough. While pouring sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus, containing sufficient 1x TAE buffer so as to cover the wells completely. Afterwards, ready to use standard DNA ladder (Thermo scientific Generuler) was loaded in first and last well of the gel which was composed of 14 double stranded DNA fragments (3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 base pairs). It also had two reference bands (1000 bp and...
500 bp). After adding 2 μl of 6x loading dye in each tube (Stock F Table 3.4) with the help of micropipette, the amplified products (25μl in each tube) to be analysed were then carefully loaded into the sample wells in between the standards. Electrophoresis was carried out at 60 volts, until the tracking dry migrated to the end of the gel. Ethidium bromide stained DNA bands were visualized under UV-transilluminator and photographed by gel documentation system. The size of the amplification products generated was estimated using molecular weight marker bands.

### 3.2.4 DATA SCORING

The data scoring method for RAPD and isozyme was same as described by Wendel and Weeden (1989). The measurement of band position for RAPD data was made by using standard DNA ladder and for isozyme data Rf value was calculated by using the following formula.

\[
R_f = \frac{\text{Distance moved by the isozyme (cm)}}{\text{Distance moved by the tracking dye (cm)}}
\]

The DNA banding patterns and the zymograms, so obtained were scored visually. The bands were scored as ‘1’ for presence and ‘0’ for absence, to generate the ‘0’ and ‘1’ data matrix.

### 3.2.5 DATA ANALYSIS

Morphological data were analysed using chi square test whers the observations were less than 10 (plant height and crown) and independent samples T test was applied to compare the means of two variables where observations were more than 30.

Unweighed pair group arithmetic mean (UPGMA) based Cluster analysis was done using Jaccard’s coefficient similarity matrix among RAPD and isozymes data marix. Similarity index (SI) between RAPD and isozyme profiles of any two individuals was calculated by using following formula:

\[
\text{Similarity Index (SI)} = \frac{2\text{NAE}}{\text{NA} + \text{NB}}
\]
Where, NAB represents total number of bands shared by individuals A and B. NA and NB are the numbers of fragments scored for each individual, respectively.

Percentage of apomixis in each mother plant was also calculated using formula:

\[
\% \text{ Apomixis} = \frac{\text{No. of progenies identical to mother plant}}{\text{Total no. of progenies of mother plant}} \times 100
\]

Percentage polymorphism, mean heterozygosity, Nei’s genetic distance and Shannon’s diversity index among different populations were also calculated using GenALEx 6.5 appendix software with 9999 permutation. Analysis of molecular variance (AMOVA) from RAPD data was used to analyze the partition of the total genetic variation between the sampled species, between and within populations.