CHAPTER-III
MATERIALS AND METHODS

The present investigation on “Molecular screening and characterization of castor genotypes for fusarium wilt resistance” was carried out at the Department of Biotechnology, Junagadh Agricultural University, Junagadh.

3.1 EXPERIMENTAL MATERIALS

The seeds of following 20 genotypes of castor were used for the present study were obtained from the Main Oilseeds Research Station, JAU, Junagadh. The genotypes are listed in Table 3.1.

Table: 3.1: List of castor genotypes

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name of the genotypes</th>
<th>Source of the genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48-1</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>2</td>
<td>JI-35</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>3</td>
<td>JI-258</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>4</td>
<td>JI-273</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>5</td>
<td>JI-368</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>6</td>
<td>JI-397</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>7</td>
<td>JI-435</td>
<td>JAU, Junagadh</td>
</tr>
<tr>
<td>8</td>
<td>SKP-84</td>
<td>SDAU, Sardarkrushinagar</td>
</tr>
<tr>
<td>9</td>
<td>SKI-346</td>
<td>SDAU, Sardarkrushinagar</td>
</tr>
<tr>
<td>10</td>
<td>RG-43</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>11</td>
<td>RG-1954</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>12</td>
<td>RG-2364</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>13</td>
<td>RG-2800</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>14</td>
<td>RG-3041</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>15</td>
<td>RG-3105</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>16</td>
<td>RG-3535</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>17</td>
<td>RG-3748</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>18</td>
<td>RG-3788</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>19</td>
<td>RG-3794</td>
<td>IIOR, Hyderabad</td>
</tr>
<tr>
<td>20</td>
<td>PCS-124</td>
<td>Palem</td>
</tr>
</tbody>
</table>
3.2 GLASSWARES AND POLYWARES

The glasswares and polywares used were of standard make such as Corning or Borosil. All glasswares were scrubbed and washed thoroughly with the detergent then rinsed with tap water followed by distilled water. Finally, it was dried in an oven before use. All polywares were thoroughly cleaned as stated above and air-dried before use.

3.3 CHEMICALS

All the chemicals used in the experiment were of analytical grade from standard manufacturers like Sigma-Aldrich, E-Merck, Hi-media, Qualigenes and SISCO Research Lab. (SRL) etc. In case of fine chemicals, molecular biological grade were used, which were obtained from Merck bioscience, Bangalore.

3.4 EQUIPMENTS

The important equipments and instruments have been used are listed below:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighing Balance</td>
<td>Citizen, CX 120</td>
</tr>
<tr>
<td>Hot water Bath</td>
<td>Nova</td>
</tr>
<tr>
<td>Gel Electrophoresis unit</td>
<td>Genetix</td>
</tr>
<tr>
<td>Gel Documentation machine</td>
<td>Upland and Gene Genius Bio Imaging System, SynGene, UK</td>
</tr>
<tr>
<td>pH meter</td>
<td>Elico</td>
</tr>
<tr>
<td>Refrigerated Centrifuge</td>
<td>Plasto, Remi (C 24), Eltek</td>
</tr>
<tr>
<td>Thermal cycler</td>
<td>Eppendorf, Applied Biosystems</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>-20°C Refrigerator</td>
<td>Operon</td>
</tr>
<tr>
<td>Hot air Oven</td>
<td>Nova</td>
</tr>
<tr>
<td>Pico drop</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Microwave oven</td>
<td>IFB</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>Samsung, Voltas</td>
</tr>
<tr>
<td>Water purification system</td>
<td>Millipore-Elix, India</td>
</tr>
</tbody>
</table>
3.5 MOLECULAR CHARACTERIZATION

3.5.1 DNA Extraction

Genomic DNA was isolated from fresh leaves by modified CTAB method as described by Doyle and Doyle (1990).

3.5.1.1 Preparation of stock solutions for reagents and buffers for DNA extraction

The reagents for DNA extraction were prepared as per the method given by Doyle and Doyle (1987). The composition and procedure for preparation of various stock solutions and buffers are given in Table 3.2.

Table 3.2: Preparation of stock solutions for DNA extraction and Agarose Gel Electrophoresis

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Chemicals /Reagents</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Preparation of Extraction buffer:</td>
<td>All the reagents except β-mercaptoethanol were dissolved in 80 ml of distilled water in a sequential manner as stated and finally volume was made to 95 ml. To dissolve all the chemicals, the solution was kept in microwave oven for about 1 min. The buffer was cooled and pH was adjusted to 8.0 with Tris-Cl. Finally β-mercaptoethanol was added and volume was made to 100 ml.</td>
</tr>
<tr>
<td></td>
<td>0.1 M Tris base: 1.21 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05 M EDTA: 1.46 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl: 2.9 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2% (w/v) polyvinylpyrrolidone-40: 1 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2% (v/v) β-mercaptoethanol: 0.2 ml</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>80 % Ethanol (100 ml)</td>
<td>80 ml of ethanol was taken and 20 ml of D/W was added, mixed well and dispensed to reagent bottle and stored at 4°C.</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform: Isoamyl alcohol (24:1) (100 ml)</td>
<td>96 ml of chloroform and 4 ml of isoamyl alcohol were mixed and stored in reagent bottle at room temperature.</td>
</tr>
<tr>
<td>4</td>
<td>Ethidium Bromide (10 mg.ml⁻¹)</td>
<td>100 mg Ethidium Bromide was added to 10.0 ml of distilled water and it was kept on magnetic stirrer to ensure that the dye is dissolved completely. It was dispensed into amber colored eppendorf tube and stored at 4°C.</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Preparation and Storage</th>
</tr>
</thead>
</table>
| 5 | 1X TE buffer, (100 ml)  
-10mM Tris (pH 8.0)  
-1mM EDTA (pH 8.0) | 121 mg of Tris-Base, 37 mg EDTA were taken and dissolved in D/W and volume was adjusted to 100 ml. The solution was autoclaved and stored at room temperature. |
| 6 | TBE buffer 5X (1 litre)  
pH 8.0 | 54.5 g of Tris-Base, 27.5 g of Boric acid were taken and dissolved in distilled water. A 20 ml of 0.5M EDTA (pH 8.0) was added. The final volume was adjusted to 1 liter by D/W and the pH was adjusted to 8.0. |
| 7 | 6X- Gel loading dye | 250 mg of bromophenol blue, 250 mg of xylene cyanol FF and 30% glycerol were added in 100 ml of D/W. |
| 8 | 5M NaCl (100 ml) | 29.2 g NaCl was taken in to beaker; 50 ml of D/W was added and mixed well. When the salts get completely dissolved, the final volume was adjusted to 100 ml. It was dispensed to reagent bottle and sterilized by autoclaving. |
| 9 | 1M Tris HCl (100 ml)  
pH 8.0 | 12.1 g Tris base was dissolved in 80 ml D/W. The pH was adjusted to 8.0 by adding concentrated HCl. A total volume was adjusted to 100 ml. It was dispensed to reagent bottle and sterilized by autoclaving. |
| 10 | 0.5M EDTA (100 ml)  
pH 8.0 | 18.6 g EDTA di Sodium salt was dissolved in 80 ml D/W. The pH was adjusted to 8.0 by adding NaOH pellets. A total volume was adjusted to 100 ml. It was dispensed to reagent bottle and sterilized by autoclaving. |

#### 3.5.1.2 Protocol for isolation of genomic DNA

1. One gram of fresh leaves of each castor genotypes was collected from the pot and crushed in liquid N\textsubscript{2} with the help of mortar and pestle.
2. Two ml DNA extraction buffer was added and the content was homogenized.
3. The homogenates were transferred to 2 ml eppendorf tubes and incubated at 65\textdegree C for 45 minutes.
4. After incubation, the tubes were centrifuged at 12000 rpm for 10 minutes and supernatant was transferred to a new eppendorf tube.
5. 500 µl chloroform: isoamyl alcohol (CIA, 24:1, v/v) was added to the tube, mixed well and centrifuged at 12000 rpm for 10 minutes and the aqueous phase was recovered in new tube.
6. 50 µl of 5 M sodium acetate was added followed by 500 µl of isopropanol and kept at -20°C for 2-3 hrs for precipitation of DNA.
7. The tubes were centrifuged at 14000 rpm, 4°C for 15 minutes and the supernatant was discarded.
8. After washing with 80% ethanol, the DNA pellet was air dried and suspended in 100 µl of Tris-EDTA buffer.

3.5.1.3 Purification of DNA
1. The extracted DNA was then subjected to an additional cleaning procedure.
2. Three µl of RNase was added to 100 µl of crude DNA preparation. It was mixed thoroughly and incubated at 37°C for 45 minutes.
3. 500 µl of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly till an emulsion was formed and centrifuged for 12 minutes at 12000 rpm.
4. Supernatant was taken, avoiding the whitish layer at interface.
5. The DNA was re-precipitated by adding double the quantity of absolute alcohol.
6. To pellet the DNA, the tubes were centrifuged at 14000 rpm, 4°C (very necessary) for 15 minutes and the supernatant was discarded.
7. The pellet was washed with 80% alcohol and air dried for 30 minutes.
8. The DNA was re-dissolved in 200 µl of TE buffer for further use.

3.5.1.4 Estimation of quantity and quality of DNA
In order to perform PCR based analysis, the DNA concentration was determined by picodrop (Qiagen). 0.2 µl sample was mounted on QIAxpert Slide-40 for measurement of quality at A260/A280 ratio which was ranged, 1.71 to 1.99 among 20 genotypes and the quantity was directly displayed as ng.µl⁻¹ that is given in Table 3.3. The concentration of DNA was adjusted to 50 ng.µl⁻¹ for further work.
Table 3.3: Purity and concentration of genomic DNA of castor genotypes

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Genotypes</th>
<th>Absorbance A_{260}/A_{280} ratio</th>
<th>Concentration ng.µl⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48-1</td>
<td>1.88</td>
<td>223.04</td>
</tr>
<tr>
<td>2</td>
<td>JI-35</td>
<td>1.78</td>
<td>325.93</td>
</tr>
<tr>
<td>3</td>
<td>JI-258</td>
<td>1.82</td>
<td>213.31</td>
</tr>
<tr>
<td>4</td>
<td>JI-273</td>
<td>1.79</td>
<td>107.04</td>
</tr>
<tr>
<td>5</td>
<td>JI-368</td>
<td>1.84</td>
<td>224.83</td>
</tr>
<tr>
<td>6</td>
<td>JI-397</td>
<td>1.83</td>
<td>136.09</td>
</tr>
<tr>
<td>7</td>
<td>JI-435</td>
<td>1.89</td>
<td>214.73</td>
</tr>
<tr>
<td>8</td>
<td>SKP-84</td>
<td>1.84</td>
<td>192.97</td>
</tr>
<tr>
<td>9</td>
<td>SKI-346</td>
<td>1.71</td>
<td>96.94</td>
</tr>
<tr>
<td>10</td>
<td>RG-43</td>
<td>1.88</td>
<td>97.98</td>
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<td>RG-1954</td>
<td>1.82</td>
<td>352.05</td>
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<td>RG-2364</td>
<td>1.78</td>
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<td>1.95</td>
<td>60.58</td>
</tr>
<tr>
<td>14</td>
<td>RG-3041</td>
<td>1.83</td>
<td>87.88</td>
</tr>
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<td>RG-3105</td>
<td>1.76</td>
<td>103.19</td>
</tr>
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<td>RG-3535</td>
<td>1.82</td>
<td>237.13</td>
</tr>
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<td>17</td>
<td>RG-3748</td>
<td>1.99</td>
<td>171.78</td>
</tr>
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<td>18</td>
<td>RG-3788</td>
<td>1.73</td>
<td>156.65</td>
</tr>
<tr>
<td>19</td>
<td>RG-3794</td>
<td>1.87</td>
<td>214.80</td>
</tr>
<tr>
<td>20</td>
<td>PCS-124</td>
<td>1.83</td>
<td>142.49</td>
</tr>
</tbody>
</table>

3.5.2 Agarose Gel Electrophoresis

To measure the integrity of DNA, agarose gel electrophoresis was used.

3.5.2.1 Chemicals used for agarose gel electrophoresis

a) Agarose (low EEO type) (Himedia, Bangalore)
b) 5-X Tris Borate EDTA (TBE) buffer pH 8.0
c) Gel loading dye (6X)
d) Ethidium bromide (10 mg.ml⁻¹)
3.5.2.2 Preparation of gel

a. Agarose gel of 0.8% was prepared (0.8 g agarose in 100 ml 1X TBE and 5.0 µl EtBr from 10 mg.ml⁻¹ stock).

b. About 10 µl of DNA + 1 µl of 6 X gel loading dye were loaded in each well.

c. The gel was run at 70 V for 1 hours and DNA bands were visualized under UV transilluminator (254nm) and photographed using Gel Documentation System.

d. Presence of single compact band on agarose gel indicated integrity of isolated DNA (Fig. 3.2).

Figure: 3.1: Electrophoretic banding pattern of genomic DNA from 20 castor genotypes on 0.8% agarose gel.


3.6. MOLECULAR MARKERS ANALYSIS OF CASTOR GENOTYPES

Various molecular marker techniques such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) were used for fingerprinting of castor genotypes. Primers required for the above techniques were synthesized from Merck bioscience, Bangalore.

All primers for RAPD, ISSR and SSR were diluted by adding equal amount of deionized sterile distilled water equal to its concentration. (e.g. if the concentration of RAPD primer is 72 nMoles then to make a concentration of 1 nMole.µl⁻¹=1000 pMoles.µl⁻¹, 72 µl of deionized water was added. This was kept as a stock solution of primer. By taking 5 µl of stoke (1000 pMoles.µl⁻¹) and 195 µl of deionized sterile distilled water a final concentration of 25 pMoles.µl⁻¹ was made. This working solution is used for PCR amplification for various molecular techniques.
3.6.1 Random Amplified Polymorphic DNA (RAPD)

Amplification of RAPD fragments was performed according to method given by Dhingani et al. (2011) with some modifications using decamer arbitrary primers (Table 3.4).

**Table 3.4: List of RAPD primers**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer Series</th>
<th>Sequence 5’- 3’</th>
<th>GC (%)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPM-02</td>
<td>5’-ACAACGCCTC-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>2</td>
<td>OPM-07</td>
<td>5’-GGCGGTGTC-3’</td>
<td>60</td>
<td>27.0</td>
</tr>
<tr>
<td>3</td>
<td>OPM-08</td>
<td>5’-TCTGTTCCC-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>4</td>
<td>OPM-10</td>
<td>5’-TTCGGCGAC-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>5</td>
<td>OPN-02</td>
<td>5’-CTCGTTCG-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>OPN-05</td>
<td>5’-ACTGACC-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>7</td>
<td>OPN-06</td>
<td>5’-AACAGGGCA-3’</td>
<td>60</td>
<td>27.0</td>
</tr>
<tr>
<td>8</td>
<td>OPN-09</td>
<td>5’-TGCCGGCTT-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>9</td>
<td>OPN-10</td>
<td>5’-ACAACATTG-3’</td>
<td>50</td>
<td>23.0</td>
</tr>
<tr>
<td>10</td>
<td>OPO-01</td>
<td>5’-GGCACGTAAG-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>11</td>
<td>OPO-03</td>
<td>5’-CTGTTGCTAC-3’</td>
<td>50</td>
<td>23.0</td>
</tr>
<tr>
<td>12</td>
<td>OPO-05</td>
<td>5’-CCACAGTACT-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>13</td>
<td>OPO-06</td>
<td>5’-CCACGGGAC-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>14</td>
<td>OPP-07</td>
<td>5’-GTCCATCCA-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>15</td>
<td>OPP-10</td>
<td>5’-TCCGCTCAG-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>16</td>
<td>OPQ-09</td>
<td>5’-GGTCACCTCA-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>17</td>
<td>OPR-08</td>
<td>5’-CCGGTCGCT-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>18</td>
<td>OPR-09</td>
<td>5’-TGAGCAGGAG-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
<tr>
<td>19</td>
<td>OPS-03</td>
<td>5’-CAGAGGCTCC-3’</td>
<td>70</td>
<td>27.0</td>
</tr>
<tr>
<td>20</td>
<td>OPS-07</td>
<td>5’-TCGATGCTG-3’</td>
<td>60</td>
<td>25.0</td>
</tr>
</tbody>
</table>

3.6.1.1 PCR reagents

The reagents used for PCR amplification of DNA were as following:

1. 10X PCR buffer [Tris (pH 9.0), KCl, 15 mM MgCl₂, Gelatin] (Merck bioscience, Bangalore)
2. Taq DNA polymerase (Merck bioscience, Bangalore)
3. dNTP mix (Merck bioscience, Bangalore)
4. Primers (25 pmoles.µl⁻¹)
3.6.1.2 PCR protocol

The mastermix was prepared in a microfuge tube in which the buffer was added first followed by sterile water, primer, dNTPs mix followed by Taq DNA polymerase (Table 3.5). At the last DNA was added in each tube separately.

**Table 3.5: Preparation of reaction mixture for RAPD**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR buffer (10X)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>2</td>
<td>Taq polymerase (3 U.μl⁻¹)</td>
<td>0.15μl</td>
</tr>
<tr>
<td>3</td>
<td>dNTPs mix (2.5 mM each)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>4</td>
<td>Primer (25 pmoles.μl⁻¹)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>5</td>
<td>Template DNA (50 ng.μl⁻¹)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>6</td>
<td>Millipore sterile distilled water</td>
<td>9.75 μl</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>15 μl</strong></td>
</tr>
</tbody>
</table>

The reagents were mixed gently by tapping against the tube. The tubes were then placed in the Thermal Cycler for amplification. The PCR condition for thermal cycler is given in Table 3.6 and Figure 3.3.

**Table 3.6: PCR conditions for RAPD**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94</td>
<td>4.0 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>1.0 min</td>
</tr>
<tr>
<td>3</td>
<td>Annealing (37-39 °C)</td>
<td></td>
<td>1.5 min</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
</tr>
<tr>
<td></td>
<td>Repeat the steps 2 to 4 for 35 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72</td>
<td>7.0 min</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4</td>
<td>--</td>
</tr>
</tbody>
</table>
3.6.1.3 Electrophoresis of amplified product

PCR products were subjected to electrophoresis with marker DNA of known molecular weight in 1.5 % agarose gel. After electrophoresis, the gel was carefully taken out of the casting tray and photographed in SynGene gel documentation system.

3.6.2 Inter Simple Sequence Repeat (ISSR)

The genomic DNA was amplified using ISSR primer series given in Table 3.7 Tomar et al. (2014).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>ISSR Primer</th>
<th>Sequence (5′→3′)</th>
<th>GC (%)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UBC-807</td>
<td>5′-(AG)$_8$T-3′</td>
<td>47.0</td>
<td>42.4</td>
</tr>
<tr>
<td>2</td>
<td>UBC-808</td>
<td>5′-(AG)$_8$C-3′</td>
<td>53.0</td>
<td>46.8</td>
</tr>
<tr>
<td>3</td>
<td>UBC-809</td>
<td>5′-(AG)$_8$G-3′</td>
<td>53.0</td>
<td>46.6</td>
</tr>
<tr>
<td>4</td>
<td>UBC-811</td>
<td>5′-(GA)$_8$C-3′</td>
<td>52.9</td>
<td>43.2</td>
</tr>
<tr>
<td>5</td>
<td>UBC-812</td>
<td>5′-(GA)$_8$A-3′</td>
<td>47.0</td>
<td>44.3</td>
</tr>
<tr>
<td>6</td>
<td>UBC-814</td>
<td>5′-(CT)$_8$A-3′</td>
<td>47.0</td>
<td>41.3</td>
</tr>
<tr>
<td>7</td>
<td>UBC-815</td>
<td>5′-(CT)$_8$G-3′</td>
<td>53.0</td>
<td>44.9</td>
</tr>
<tr>
<td>8</td>
<td>UBC-816</td>
<td>5′-(CA)$_8$T-3′</td>
<td>47.0</td>
<td>51.1</td>
</tr>
<tr>
<td>9</td>
<td>UBC-817</td>
<td>5′-(CA)$_8$A-3′</td>
<td>47.0</td>
<td>52.7</td>
</tr>
<tr>
<td>10</td>
<td>UBC-819</td>
<td>5′-(GT)$_8$A-3′</td>
<td>47.0</td>
<td>47.6</td>
</tr>
<tr>
<td>11</td>
<td>UBC-823</td>
<td>5′-(TC)$_8$C-3′</td>
<td>53.0</td>
<td>47.5</td>
</tr>
<tr>
<td>12</td>
<td>UBC-826</td>
<td>5′-(AC)$_8$C-3′</td>
<td>53.0</td>
<td>53.3</td>
</tr>
<tr>
<td>13</td>
<td>UBC-829</td>
<td>5′-(TG)$_8$C-3′</td>
<td>53.0</td>
<td>56.3</td>
</tr>
<tr>
<td>14</td>
<td>UBC-834</td>
<td>5′-(AG)$_8$YT-3′</td>
<td>44.4</td>
<td>49.8</td>
</tr>
<tr>
<td>15</td>
<td>UBC-836</td>
<td>5′-(AG)$_8$YA-3′</td>
<td>44.4</td>
<td>40.8</td>
</tr>
<tr>
<td>16</td>
<td>UBC-840</td>
<td>5′-(GA)$_8$YT-3′</td>
<td>44.4</td>
<td>52.9</td>
</tr>
<tr>
<td>17</td>
<td>UBC-841</td>
<td>5′-(GA)$_8$YC-3′</td>
<td>50.0</td>
<td>45.7</td>
</tr>
<tr>
<td>18</td>
<td>UBC-842</td>
<td>5′-(AG)$_8$YG-3′</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>19</td>
<td>UBC-845</td>
<td>5′-(CT)$_8$RC-3′</td>
<td>50.0</td>
<td>43.4</td>
</tr>
<tr>
<td>20</td>
<td>UBC-847</td>
<td>5′-(CA)$_8$RC-3′</td>
<td>50.0</td>
<td>46.0</td>
</tr>
</tbody>
</table>
The PCR reactions for ISSR were prepared according to method given by Williams et al., (1990) with some modifications for PCR conditions (Table 3.8 and Table 3.9).

### 3.6.2.1 PCR protocol

The master mixture was prepared in a microfuge tube in which the buffer was added first followed by sterile water, primer, dNTPs mix followed by Taq DNA polymerase (Table 3.8). At the last DNA was added in each tube separately.

**Table 3.8: Preparation of reaction mixture for ISSR**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR buffer (10X)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>2</td>
<td>Taq polymerase (3 U.μl⁻¹)</td>
<td>0.15μl</td>
</tr>
<tr>
<td>3</td>
<td>dNTPs mix (2.5 mM each)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>4</td>
<td>Primer (25 pmoles.μl⁻¹)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>5</td>
<td>Template DNA (50 ng.μl⁻¹)</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>6</td>
<td>Millipore sterile distilled water</td>
<td>9.75 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>15 μl</strong></td>
</tr>
</tbody>
</table>

The reagents were mixed gently by tapping against the tube. The tubes were then placed in the Thermal Cycler for amplification. The PCR condition for thermal cycler is given in Table 3.9 and Figure 3.4.

**Table 3.9: PCR conditions for ISSR**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95</td>
<td>5.0 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>1.0 min</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>50 °C</td>
<td>1.0 min</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>2.0 min</td>
</tr>
<tr>
<td></td>
<td>Repeat the steps 2 to 4 for 35 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72</td>
<td>5.0 min</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4</td>
<td>--</td>
</tr>
</tbody>
</table>
Materials and Methods

Figure 3.3: Steps in ISSR PCR amplification

3.6.2.2 Electrophoresis of amplified product

PCR products were subjected to electrophoresis with marker DNA of known molecular weight in 1.5 % agarose gel. After electrophoresis, the gel was carefully taken out of the casting tray and photographed in SynGene gel documentation system.

3.6.3 SIMPLE SEQUENCE REPEATS (SSRs)

The genomic DNA was amplified using SSR primer series given in Table 3.10 Sakure et al. (2012).

Table 3.10: List of SSR primers

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>GC (%)</th>
<th>Tm (°C)</th>
<th>Annealing Tm for PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Castor 5</td>
<td>F: CGTGAACAGGGACGATATTCC</td>
<td>50</td>
<td>61.9</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTCACGCGCTTCTTGCCTC</td>
<td>55.5</td>
<td>65.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Castor 6</td>
<td>F: GGGCAAATACATGAGGCG</td>
<td>50</td>
<td>62.1</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCAGGACTCACAGGGAGAGA</td>
<td>55</td>
<td>62.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Castor 7</td>
<td>F: AAAAGGAAAAAGAAAAACCTC</td>
<td>45</td>
<td>62.3</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATTAAGGAGTTTGGGTT CGG</td>
<td>45</td>
<td>61.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Castor 8</td>
<td>F: CTTTTCTCCCTCAATGAGAT</td>
<td>45</td>
<td>61.8</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TATAGAGTTGGAGGTGC GG</td>
<td>50</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Castor 18</td>
<td>F: CCGAAGAATGGGAAAGTTAT</td>
<td>45</td>
<td>61.6</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGATCTTCAGGTAACGGAGGC</td>
<td>50</td>
<td>61.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Castor 19</td>
<td>F: GGTCGGGTTGAGGACTTAT</td>
<td>50</td>
<td>61.7</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCATACCTATAGTCACG CGG</td>
<td>55</td>
<td>61.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Castor 20</td>
<td>F: AGAGGCCGCCACACTACAAC</td>
<td>50</td>
<td>61.2</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGAGGGCCCACCATACAAC</td>
<td>55</td>
<td>61.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Castor 25</td>
<td>F: AGCATATTTCGGGAGAACAG</td>
<td>45</td>
<td>61.8</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCGATTCAGGTAACGGAGGC</td>
<td>50</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Castor</td>
<td>F:</td>
<td>R:</td>
<td>Tm</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------</td>
<td>----------</td>
<td>----------</td>
<td>-----</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>AAATAAGAAACCCTAGCCCG</td>
<td>AAATAAGAAACCCTAGCCCG</td>
<td>45</td>
<td>60.9</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>CTTGCAACGGGACGATATTC</td>
<td>CTTGCAACGGGACGATATTC</td>
<td>45</td>
<td>61.9</td>
</tr>
<tr>
<td>11</td>
<td>38</td>
<td>CTTCATATCCGAGCTCTCT</td>
<td>CTTCATATCCGAGCTCTCT</td>
<td>45</td>
<td>61.8</td>
</tr>
<tr>
<td>12</td>
<td>42</td>
<td>ATGACCTCCATCTCCACAA</td>
<td>ATGACCTCCATCTCCACAA</td>
<td>45</td>
<td>62.0</td>
</tr>
<tr>
<td>13</td>
<td>45</td>
<td>GCCATTTGTCACCATGTCTC</td>
<td>GCCATTTGTCACCATGTCTC</td>
<td>45</td>
<td>62.2</td>
</tr>
<tr>
<td>14</td>
<td>48</td>
<td>TGATCTTCAGGTAACGAGGC</td>
<td>TGATCTTCAGGTAACGAGGC</td>
<td>45</td>
<td>61.8</td>
</tr>
<tr>
<td>15</td>
<td>51</td>
<td>CCGAAGAATGGAAGGTATAT</td>
<td>CCGAAGAATGGAAGGTATAT</td>
<td>45</td>
<td>61.6</td>
</tr>
<tr>
<td>16</td>
<td>53</td>
<td>TCTGCAACCACTAGCTCC</td>
<td>TCTGCAACCACTAGCTCC</td>
<td>45</td>
<td>61.8</td>
</tr>
<tr>
<td>17</td>
<td>62</td>
<td>TTACCATAACATCACATCCAC</td>
<td>TTACCATAACATCACATCCAC</td>
<td>45</td>
<td>62.0</td>
</tr>
<tr>
<td>18</td>
<td>72</td>
<td>GGGCACGATGTGAGAGTTTA</td>
<td>GGGCACGATGTGAGAGTTTA</td>
<td>45</td>
<td>61.8</td>
</tr>
<tr>
<td>19</td>
<td>73</td>
<td>CTTGCTCTCCATCGTTTCTCT</td>
<td>CTTGCTCTCCATCGTTTCTCT</td>
<td>45</td>
<td>61.2</td>
</tr>
<tr>
<td>20</td>
<td>78</td>
<td>GAAGCTTGAATGAGCAGGGACG</td>
<td>GAAGCTTGAATGAGCAGGGACG</td>
<td>45</td>
<td>61.9</td>
</tr>
<tr>
<td>21</td>
<td>82</td>
<td>TCCAAACGCGCCACAAA</td>
<td>TCCAAACGCGCCACAAA</td>
<td>45</td>
<td>62.0</td>
</tr>
<tr>
<td>22</td>
<td>97</td>
<td>CGGATCCAATCTAAAGACGG</td>
<td>CGGATCCAATCTAAAGACGG</td>
<td>45</td>
<td>61.9</td>
</tr>
<tr>
<td>23</td>
<td>98</td>
<td>ATAGCCTCTCGAGACGAGGG</td>
<td>ATAGCCTCTCGAGACGAGGG</td>
<td>45</td>
<td>61.9</td>
</tr>
<tr>
<td>24</td>
<td>111</td>
<td>TGAGCAGCTCTCGATGCTCTC</td>
<td>TGAGCAGCTCTCGATGCTCTC</td>
<td>45</td>
<td>61.4</td>
</tr>
<tr>
<td>25</td>
<td>114</td>
<td>AGCAGCTATCAGTCTCTT</td>
<td>AGCAGCTATCAGTCTCTT</td>
<td>45</td>
<td>62.4</td>
</tr>
<tr>
<td>26</td>
<td>117</td>
<td>TTGTAATCTCCACTCTCAGCC</td>
<td>TTGTAATCTCCACTCTCAGCC</td>
<td>45</td>
<td>62.1</td>
</tr>
<tr>
<td>27</td>
<td>123</td>
<td>AGTTCGGACTCTCTTTGCAC</td>
<td>AGTTCGGACTCTCTTTGCAC</td>
<td>45</td>
<td>62.0</td>
</tr>
</tbody>
</table>
3.6.3.1 PCR Protocol

The mastermix was prepared in a microcentrifuge tube in which the buffer was added first followed by sterile water, primer, dNTPs mix followed by Taq DNA polymerase (Table 3.11). At the last DNA was added in each tube separately.

**Table 3.11: Preparation of reaction mixture for SSR**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR buffer (10X)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>2</td>
<td>Taq polymerase (3 U.µl⁻¹)</td>
<td>0.15 µl</td>
</tr>
<tr>
<td>3</td>
<td>dNTPs mix (2.5 mM each)</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>4</td>
<td>Primer-F (25 pmoles.µl⁻¹)</td>
<td>0.6 µl</td>
</tr>
<tr>
<td>5</td>
<td>Primer-R (25 pmoles.µl⁻¹)</td>
<td>0.6 µl</td>
</tr>
<tr>
<td>6</td>
<td>Template DNA (50 ng.µl⁻¹)</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>7</td>
<td>Millipore sterile distilled water</td>
<td>9.75 µl</td>
</tr>
</tbody>
</table>

**Total** 15 µl

The reagents were mixed gently by tapping against the tube. The tubes were then placed in the Thermal Cycler for amplification. The PCR condition for thermal cycler is given in Table 3.12 and Figure 3.5

**Table 3.12: PCR conditions for SSR**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94</td>
<td>4.0 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>45 sec</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>(58- 60) °C</td>
<td>45 sec</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
</tr>
</tbody>
</table>

Repeat the steps 2 to 4 for 35 times

| 5      | Final extension   | 72               | 7.0 min  |
| 6      | Hold              | 4                | --       |
3.7 STATISTICAL ANALYSIS FOR MOLECULAR PARAMETERS

3.7.1 Dendrogram Analysis

Clear and distinct bands amplified by RAPD, ISSR and SSR primers were scored for the presence (1) and absence (0) for the corresponding band among the genotypes. The data were entered into MS-Excel data sheet and subsequently analyzed using NTSYS-pc version 2.02 (Rohlf, 1998).

3.7.2 Polymorphic Information Content (PIC) Calculation

Polymorphic information content (PIC) for RAPD, ISSR and SSR was calculated on the basis of allele frequency (Anderson et al., 1993).

\[ \text{PIC}_i = 1 - \sum_{j=1}^{n} P_{ij}^2 \]

Where \( P_{ij} \) is the frequency of \( j^{th} \) allele for marker \( i \), and summation extends over \( n \) alleles.

PIC values were then used to calculate a RAPD primer index (RPI), ISSR primer index (IPI) and SSR primer index (SPI), which were generated by multiplying the PIC values of all the markers amplified by the same primer.

The data matrix was read by NTSYS-pc version 2.02 (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Exeter Software) and
analyzed by the SIMQUAL (Similarity for Qualitative Data) program with Jaccard’s similarity coefficient. SIMQUAL is a program for computing a variety of similarity and dissimilarity coefficients for qualitative data. The qualitative nature of the absence (0) or presence (1) state of a molecular marker was used as the basis for similarity analysis among various castor genotypes. A matrix of 0 and 1 act as the input and the output is a matrix of similarity or dissimilarity coefficients. The resultant similarity matrix was entered into SAHN (Sequential, Agglomerative, Hierarchical and Nested clustering method) clustering program, a tree matrix was produced and a dendrogram constructed using UPGMA (Unweighted Pair-Group Method with Arithmetic Averages). The assumption underlying the use of UPGMA clustering is the equal rate of evolution along all dendrogram branches. Dendrogram of publication quality were produced from the output tree file of SAHN by TREE (Tree Display) program in graphics mode.

Clustering methods create clusters of the data, no matter whether there are true clusters in the data or not, so a check was made for the existence of true clusters. This was done by using the tree matrix produced by SAHN to calculate the cophenetic values of similarity or dissimilarity by the program COPH (Cophenetic Values). The cophenetic value matrix was compared with the original tree matrix for goodness of fit of the cluster analysis to the data. This type of cophenetic correlation was done by the MXCOMP (Matrix Comparison) program (Rohlf 1998). The program MXCOMP plots the cophenetic value matrix against the original tree matrix and computes the cophenetic correlation coefficient (r) and the Mantel test statistic (Z).

The test criterion of mantel test is as follows:

\[
Z = \sum_{i < j} X_{ij}Y_{ij}
\]

\[
X_{ij} = \text{off-diagonal elements of cophenetic value matrix}
\]

\[
Y_{ij} = \text{off-diagonal elements of original tree matrix}
\]

\[
n = \text{the number of elements of the matrices}
\]

As the cophenetic correlation coefficient is positively correlated to the Mantel test statistic and in standardized units, it is easier to use it as a measure of goodness of fit for a cluster analysis than the Mantel test statistic. The degree of fit can be referred as follows (Rohlf, 1998):
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<table>
<thead>
<tr>
<th>Level</th>
<th>Degree of Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.9 \leq r$</td>
<td>Very good fit</td>
</tr>
<tr>
<td>$0.8 \leq r &lt; 0.9$</td>
<td>Good fit</td>
</tr>
<tr>
<td>$0.7 \leq r &lt; 0.8$</td>
<td>Poor fit</td>
</tr>
<tr>
<td>$r &lt; 0.7$</td>
<td>Very poor fit</td>
</tr>
</tbody>
</table>

3.8 SCREENING OF CASTOR GENOTYPES FOR FUSARIUM WILT RESISTANCE

Experiment was carried out in pot with equal weight of soil. Twenty genotypes of castor were sown in Net House, Department of Biotechnology, College of Agriculture, JAU, Junagadh. The inoculum of various isolates *Fusarium oxysporum f.sp. ricini* was prepared on half boiled jowar media. The flasks containing the sterilized media was inoculated with mycelial disc of *Fusarium oxysporum f.sp.ricini* (5 mm diameter) and incubated at 27-29°C for 10 days. This inoculum was used for soil inoculation at 40 g per kg soil in all the pots except control. The pathogenic nature of the organism was proved by using Koch’s postulates. Germinated seeds were counted after 10 to 14 days of sowing. The symptoms of wilt disease of castor and expression of the characteristic symptoms and variation were recorded. Screened genotypes were grouped in resistant and susceptible groups.

**Table 3.13:** Composition of Potato Dextrose Agar:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato Infusion</td>
<td>200 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

3.8.1 Disease incidence

Disease incidence was measured by assessing the percentage of dead plants or the length of stem lesion (Anjani *et al.*, 2004). Resistant and susceptible genotypes were screened for fusarium wilt resistance.

Per cent (%) disease incidence

$$= \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$