3. MATERIALS AND METHODS

The present investigation was carried out in the Department of Plant Pathology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur. The materials and the methodology adopted to achieve the objectives of the investigation are given here under:

3.1 Development of mapping population

3.1.1 Plant material

The F$_2$ seeds of Jawala cross KRC5 population were obtained from Department of Plant Pathology and were subjected to phenotypic evaluation to study genetics of resistance and development of RIL mapping population. Landrace KRC5 in Jawala x KRC5 cross is resistance gene source (donor) and Jawala being a well-adapted commercial variety used as susceptible parent (recipient). The resistant and susceptible nature of individuals was confirmed by inoculating 10 plants of each line with each of three of C. lindemuthianum races. The agronomic traits and reaction on both the parent genotypes were recorded and in table 3.1.

### Table 3.1. Agronomic characteristics of parental genotypes

<table>
<thead>
<tr>
<th>Accession</th>
<th>Growth habit</th>
<th>Colour</th>
<th>Seed size</th>
<th>Disease reaction</th>
<th>Gene pool</th>
<th>Maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jawala</td>
<td>I</td>
<td>Dark red</td>
<td>Large</td>
<td>Susceptible</td>
<td>Andean</td>
<td>Early</td>
</tr>
<tr>
<td>KRC5</td>
<td>II</td>
<td>Purple</td>
<td>Large</td>
<td>Resistant</td>
<td>Andean</td>
<td>Late</td>
</tr>
</tbody>
</table>

*I*: Determinate type; II Indeterminate type

3.1.2 Development of recombinant inbred lines (RILs)

An F$_2$ population derived from the cross between KRC5 as a male (resistant parent) with the susceptible cv. ‘Jawala’ was obtained from Department of Plant pathology CSK HPKV. One hundred F$_{2;7}$ RILs mapping population were developed by single seed descent advancement (SSD) of F$_2$ at the CSK HPKV, Palampur and MAREC, Sangla, Kinnaur (HP).
Fig 3.1. Development of recombinant inbred lines (RILs) mapping population of the cross between Jawala and KRC-5
3.2 Phenotypic evaluation of common bean segregating populations/ genotypes with test *C. lindemuthianum* races

3.2.1 Isolation and maintenance of *C. lindemuthianum* races

Cultures of three *C. lindemuthianum* races viz., 3, 537 and 935 maintained as infected hypocotyl bits of susceptible cultivar ‘Jawala’ at 4°C in the Department of Plant Pathology were revived on Mathur’s medium. Infected sample bits of 2-3 mm diameter were surface sterilized by dipping in a solution of 0.1 per cent mercuric chloride for 10-15 seconds and then washed with sterilized distilled water twice to remove traces of mercuric chloride under laminar air flow. The bits were dried under two folds of sterilized filter papers and transferred to Mathur’s medium slants aseptically with the help of inoculation needle in a laminar air flow. Slants were incubated in a B.O.D incubator at 22 ± 2°C for 7-8 days.

Each isolate was purified by taking single spore purified using standard serial dilution technique incubated at 24°C for 4 hours on 2 per cent water agar. Pathogen cultures were maintained on Mathur’s medium. To ensure continued pathogenicity, the fungus was re-inoculated onto susceptible bean plants every six months and then re-isolated a fresh. For those isolates which were not required for frequent use, were stored on PDA at 4°C for longer periods without re-isolation.

3.2.2 Confirmation of race identity

Identity of purified cultures of three races was confirmed by inoculating each on a set of bean differential cultivars (Michelite, MDRK, Perry marrow, Cornell 49.242, Widusa, Kaboon, Mexico 222, PI 207262, TO, TU, AB 136, G2333) which are internationally recommended for identification and classification of unknown *C. lindemuthianum* isolates into races (Drijfhout and Davis 1989; Pastor-Corrales 1991; Sharma et al. 1999).

3.2.3 Inoculum preparation

Each multiplied single spore isolates were placed on fresh Mathur's agar medium in a Petri dish and incubated at a controlled temperature of between 22-25°C for 7-10 days to allow the fungus for enough time to produce conidial spores. The concentration of the suspension was determined using a haemocytometer. Spore density of 1.2 x 10^6
conidia per ml (Balardin and Kelly 1998; Bigirimana and Hofte 2001) obtained was then used for plant inoculations. By using the prepared inoculum, the different isolates were suspended in distilled water with a few drops of Tween 20 (0.1% v/v).

Table 3.2.  Anthracnose differential series, resistance genes, host gene pool, and the binary number of each differential cultivar, used in the characterization of races of Colletotrichum lindemuthianum, in common bean

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Differential line</th>
<th>Known gene</th>
<th>Donor variety</th>
<th>Gene Pool</th>
<th>Binary number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. A</td>
<td>Co-11</td>
<td>Michelite</td>
<td>MA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2. B</td>
<td>Co-1, Co-3</td>
<td>MDRK</td>
<td>A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3. C</td>
<td>Co-1, Co-3, Co-11</td>
<td>Perry Marrow</td>
<td>A</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4. D</td>
<td>Co-2</td>
<td>Cornell 49-242</td>
<td>MA</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5. E</td>
<td>Co-1, Co-3, Co-2</td>
<td>Widusa</td>
<td>A</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>6. F</td>
<td>Co-1</td>
<td>Kaboon</td>
<td>A</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>7. G</td>
<td>Co-3</td>
<td>Mexico 222</td>
<td>MA</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>8. H</td>
<td>Co-4, Co-3/Co-9</td>
<td>PI 207262</td>
<td>MA</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>9. I</td>
<td>Co-4</td>
<td>TO</td>
<td>MA</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>10. J</td>
<td>Co-5</td>
<td>TU</td>
<td>MA</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>11. K</td>
<td>Co-6, Co-8</td>
<td>AB 136</td>
<td>MA</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>12. L</td>
<td>Co-4, Co-5, Co-7</td>
<td>G 2333</td>
<td>MA</td>
<td>2048</td>
<td></td>
</tr>
</tbody>
</table>

2 Known host resistance Co- genes; the resistance gene(s) in ‘Michelite’ and ‘Widusa’ have not been fully characterized. X MA = Middle American gene pool; A = Andean gene pool of the host (Gepts 1988). X Binary number: for 2^n is equivalent to the place of the differential cultivar within the series. The sum of cultivars with susceptible reaction will give the binary number of a specific race (Melotto and Kelly 2000; Pastor-Corrales 1991).

3.2.4 Method of inoculation

Germinating seed dip method as described by Champion et al. 1973 was used for inoculation to study the reaction type of test isolates on differential cultivar and evaluation of resistance against race 3 of pathogen. Seeds were germinated by rolled towel method. Surface sterilized healthy seeds were placed in double layer of moistened seed germination paper
at 26-28°C in seed germinator with 12 hour photoperiod for three days. Seed coat of germinated seeds was removed and dipped in standard suspension for about 5 minutes. Inoculated seeds were sown 3 cm deep in plastic trays (30 x 15 cm² size) filled with sterilized sand and incubated at 22 ± 1 °C with more than 90 per cent relative humidity in growth chamber (Drijfhout and Davis 1989). Germinated seeds dipped in inoculated with distilled water served as control. Observation on disease severity was recorded after 6 th and 12 th day of inoculation.

3.2.5 Inheritance of resistance

One hundred F₂ seeds of Jawala × KRC5 were inoculated by germinating seed dip method to determine inheritance of resistance against three C. lindemuthianum races viz., 3, 537 and 935. To develop mapping population for anthracnose resistance gene, a total of 512 F₂ and 100 F₂:7 RILs seedlings of cross Jawala x KRC 5 were inoculated with race 3 of C. lindemuthianum. The number of resistant and susceptible seedlings was counted and the data was subjected to Chi-square analysis to test the goodness of fit to Mendelian ratios.

3.2.6 Evaluation of disease reaction

The disease reaction on differential cultivar/ mapping population inoculated by germinating seed dip method was recorded by following 0-5 point scale given by Drijfhout and Davis 1989). The race or pathotype number was distinguished numerically as the sum of the binary values assigned to differential cultivars on which the isolate was pathogenic.

**Description of Reaction Types: Description**

0: No infection

1: Pin headpoint lesions on stem/leaves,

2: Around 3 mm sized lesions but no sunken spots and no sporulation

3: Large sunken lesions more than 3 mm size but no sporulation

4: Large sunken lesions on stem upto stem center along with sporulation

5: Seedlings killed by the pathogen.

The reaction type 0, 1 and 2 were categorized as resistant and 3, 4 and 5 were graded as susceptible.
Fig 3.2. Disease scale (0-5) for recording disease reactions of bean anthracnose

3.3 Chi- square analysis

The number of resistance and susceptible seedlings was counted and the data was subjected to Chi square analysis to test the goodness of fit to a Mendelian ratio. ‘Goodness of fit’ of the observed F$_2$ ratio with the expected Mendelian ratio was listed as

\[ 2 (n - 1) d. f = \frac{(O_i - E_i)^2}{E_i} \]

Where, \( O_i = \) Observed frequency of \( i^{th} \) class

\( E_i = \) Expected frequency of \( i^{th} \) class

\( N = \) Number of phenotypic class

In null hypothesis (H$_o$), no difference between the observed and expected frequency was accepted \( i.e. \) if the calculated value was found to be less than table value for \( (n-1) \) degree of freedom at \( P = 0.05 \) acceptance of hypothesis exist and if, it exceeds the table value, the null hypothesis was rejected and fitness to an alternative ratio was listed.
3.4 Molecular mapping of anthracnose resistance gene

Recessive class analysis (RCA) proposed by Zhang et al. 1994 was used to map the resistance gene in this study because of the reliable reactions (diagnostic symptoms) and high efficiency to locate the gene on its chromosome (Zhang et al. 1994; Lin et al. 1996; Miyamoto et al. 1996). A final mapping population of 199 plant comprised of 100 F₂ and 59 RIL susceptible to race 3 and 40 F₂ resistant plants were used to map the resistance gene tentatively designated as Co-ind.

3.4.1 Extraction of plant genomic DNA

Genomic DNA of parents and individual F₂ plants was isolated using the CTAB method of Murray and Thompson (1980). Young leaves were harvested from plants, rinsed with deionized water and dried on tissue paper. About 300 mg of fresh leaf tissue was taken from each plant and ground into fine powder using autoclaved mortar and pestle in the presence of liquid nitrogen. The powder was transferred to eppendorf tube containing 800 µl of extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl and 1% PVP, pH 8.0), maintained at 60 °C and mixed vigorously. The tubes were then incubated at 60 °C in water bath for 1 h with gentle shaking. Equal volume of chloroform: isoamyl alcohol (24:1) was added to each tube and mixed gently before centrifugation. After centrifugation at 10,000 g for 10 min at 4 °C, the upper layer (aqueous phase) was transferred to eppendorf tubes and 2 µl of RNase (10 mg/ml) was added to each tube. After adding RNase, the eppendorf tubes were incubated at 37 °C for 1 h. After RNase treatment, 600 µl of pre-chilled isopropanol was added, gently mixed with the aqueous phase and kept at -20 °C for 30 min. DNA was precipitated by centrifugation at 10,000 g for 10 min. The supernatant was drained and pellet was retained. The pellet was washed twice with 300 µl of chilled 70 per cent ethanol. The pellet was dried in a laminar air flow cabinet for 3 to 4 h. Dried DNA pellet was dissolved in 100 µl TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The yield and quality of DNA was checked by agarose gel electrophoresis using 1.2 per cent gel. The final concentration of DNA in working solution was adjusted to 10 ng/µl.
3.4.2 Selection of potential markers for PCR amplification

(A) Polymorphism survey of parents with RAPD and SSR

A total of 287 oligonucleotide RAPD primers (Operon Technologies) were will be screened with DNA of Jawala and KRC5 to select polymorphic primers showing consistent banding pattern. These includes RAPD primer as OPA (1-20), OPD (1-20), OPF (1-20), OPG(1-20), OPI(1-20), OPQ(1-20), OPU(1-20), OPV(1-20), OPW( 1-20), OPX(1-20), OPY(120), OPZ(1-20), OPAB(1-20), OPAE(1-20), OPAI(1–20), OPAP(1-20), OPAW (1-20), OPAW(1-20), OPB(4,5,6,8,10,11,20), OPC(1,2,4,7,9, 11), OPE( 8,9,18), OPH(1,3,5), OPI( 7,14,16), OPO(2,5,7,10,20), OPR(10,11,13,15), OPK(1-10), OPL(1-10,17), S(141-160), S1001-S1020, S1472, OPS11, OPT16, OPAG (1,2,3,8,9,11,13).

Table 3.3. PCR reaction mixture and amplification conditions used with different molecular markers

<table>
<thead>
<tr>
<th>Reaction constituent</th>
<th>SSR (µl)</th>
<th>RAPD (µl)</th>
<th>ScOPF6 SCAR (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer (20 mM Tris HCl, pH 8.0, 50 mM KCl)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂ (1.5 mM)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTP mix (0.2 mM) (Banglore Genei, India),</td>
<td>0.4</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Taq DNA polymerase (Bangalore Genei, India, 5 U/µl)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template (20 mg)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Forward Primer (10 mM)</td>
<td>0.8</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Reverse Primer (10 mM)</td>
<td>0.8</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Sterilized distilled water</td>
<td>16.8</td>
<td>15.8</td>
<td>16.8</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>
Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India) before running PCR Programme.

**PCR Programme:** Amplifications were performed using thermal cycler (Eppendorff Gradient PCR) programmed as given below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Primer</th>
<th>ScOPF6 SCAR (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SSR</td>
<td>RAPD</td>
</tr>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (Mins)</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>2</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>0.3</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Cycles</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10</td>
</tr>
</tbody>
</table>

A total of 212 SSR primers comprising both genic and genomic markers were used for polymorphism survey of parental isolates Jawala and KRC5. These genic/genomic primers includes primers viz., BMd (Blair et al. 2012); BMb (Cordoba et al. 2010); PV (Caixeta et al. 2005); PV (Yu et al. 2000); BMd (Blair et al. 2003); BMa, CAC (Blair et al. 2008); BM, GATS, AG (Gaitan-Solis et al. 2002).

Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India). Amplifications were performed using thermal cycler (Eppendorff Gradient PCR GeneAmp PCR system 9700, Applied Biosystems, USA).

(B) **Agarose gel electrophoresis**

The amplified PCR products were resolved by electrophoresis using 1.5 per cent agarose gel in 0.5X TAE buffer containing ethidium bromide (0.5 µg/ml) using submarine horizontal agarose slab gel (Scie-Plas) apparatus at 80 volts for 2 hr. Lambda DNA/EcoR1/HindIII double digest 1000 bp DNA ladder (MBI Fermentas, USA) and 100 bp ladder were used as standard marker and product size was viewed under Gel
documentation system using UV light and image was captured.

(C) Polyacrylamide Gel Electrophoresis (PAGE)

The silver staining protocols used here have been optimized by modifying and combining various previous versions polyacrylamide, particularly the gel silver staining protocol(s) which is as follows:

(I) Glass plates preparation

1. A pair of glass plates was selected comprising one notched plate and one unnotched gel plate.

2. Both the plates were cleaned using 70 per cent ethanol and wiped with fur free wipe across and up and down three times.

3. Bind silane was applied to the gel plate. For this, mixture of 1.1 ml 95 per cent Ethanol: 0.5 per cent Acetic Acid solution with 2.0 µl Bind silane in an eppendorf tube was prepared. This mixture was applied to the to the surface of the plate and wiped very quickly as evenly as possible. Let the glass dry for 5 minutes. Gloves were changed to avoid contamination.

5. The inner side of notched glass plate was treated with 2-3 sprays of Serva (Blueslick) and glass plate was wiped vertically and horizontally quickly with Kim-wipes. Let it dry for 5 minutes.

6. The glass plate unit was assembled and place back on the bench with treated face up. The water soaked spacers were laid on both sides of the back glass (gel plate).

7. The notched plate was carefully placed, treated side down facing the gel plate on top of the bottom assembly. Work was carried from the bottom corner, the clamps were placed on both sides of the corner to firmly hold the glass plates together assuring a tight seal. Clamps were placed on both sides of the glass unit.

8. The glass plate unit was stranded upright using the clamps as base and locked them with clips. The grooved surface was kept facing up.

9. The acryl amide gel mixture was poured slowly and carefully between the glass plates upto the top using 50 ml syringe with no bubbles trapped.
10. The inverted comb was inserted allowing the trapped bubbles to escape. Wait 1 hour for the gel to polymerize.

11. The clamps and combs were gently removed after the gel has completely polymerized.

12. The excess of polymerized gel was flush out with mild scratching and the remaining gel traces were removed by washing the groove with slight pressure using wash bottle. Thereby preparing uniform gel surface for holding comb.

13. The gel unit was held firmly from both sides and the unit was placed carefully in the bottom reservoir which has been filled with approximately 300 ml 0.5 X TBE solution notched plate facing inward. The upper buffer reservoir was closed and filled to the top with 1X TBE solution to cover the top of the gel. Care was taken that no buffer leaked from the upper reservoir.

(II) Gel Loading

1. The gel was warm up at 80-90W for 1hour.

   **Note:** Another commonly used, and preferred, option is to heat the buffer (2000ml) in the microwave on high power for 5 min.

2. 4µl of formamide loading dye was added to 10µl of reaction solution.

3. Denatured the PCR reactions for 5 min at 95ºC or boiling and immediately put into ice.

4. 4.5 µl of sample was loaded using Hamilton syringe on each lane of 6 per cent non-denaturing PAGE and as the base pair marker, 100bp DNA ladder was loaded.

   **Note:** This is a good time to make fix/stop, silver staining and developing solutions and place them in the freezer (-20ºC); the colder the solutions the better the quality of bands.

5. After loading the sample, the upper safety cover was attached. The unit was connected to the power supply with proper polarity: black leads connected to the black cathode and the red leads connected to the red anode. The power pack was turned on, and the voltage was set to 800V, and allowe the gel to run for 2.5 hours. Monitored the progress of electrophoresis following the migration of the dye.
6. When the electrophoresis is completed, the power pack was turned off and both power cords from the power supply were disconnected. The top reservoir was drain by unclamping the binder clip on the clear plastic tube allowing the TBE solution to drain into a beaker. When all of the solution has been drained, the top was removed by holding the cover.

(III) Silver Staining

1. The plates were separated while keeping the gel attached to outer glass plate.

2. The gel was fixed by placing it in cold fix/stop solution in tray (do not pour directly onto the gel) and gently agitated (place on the orbital shaker) for 20 minutes. Gel may be stored in fix/stop solution overnight (however, bands get fatter when you do this). Fix/stop solution was save and placed back in the freezer.

3. The gel was wash and rinsed three times in 1litre for 5-10 min each in ddH₂O (double distilled water) using agitation. The gel was lifted and allowed to drain 10-20 seconds.

4. Staining of the gel was done by pouring the staining solution into the tray. The gel was transferred to staining solution and agitated for 30 min. The gel was remove from tray and kept upright. In the last 10 min, the final reagents were added to the developing solution.

5. The gel tray was rinsed by approx. 1 lit of ddH₂O. The gel was submerged and agitated for 3-7 seconds only. Note: Too much time in the rinse will result in weak staining, the gel was remove from the tray and kept upright.

6. The gel was developed by pour 1L of developing solution into the tray. The gel was place in the tray and agitated until bands began to appear.

**Note:** This is a temperature sensitive reaction. The colder the solution the longer it will take for the bands to appear but the clearer they will be; a slushy consistency produces the sharpest bands. Pour in the rest of the chilled developing solution and keep agitating until band are clearly visible

**Note:** Do not forget the gel will continue to get darker until neutralize the developing solution
7. In order to neutralize the gel 2L of fix/stop solution was added directly to developing solution and agitated for 2-3 min.

   **Note:** use your hands and pull the plate up and down to more quickly neutralize the developing solution will prevent the gel from getting too dark.

8. The gel was rinsed twice using agitation for 2-3 min in 1 litre of ddH2O.

9. The gel was dried by standing the plate upright.

10. After observing on light box or scan of gel, glass plates must be cleaned up by soaking the gel plate in 3 per cent sodium hydroxide solution. After soaking for few hours a razor blade was used to remove the gel from gel plate.

   **Note:** The short glass plate should not be soaked unless it has been contaminated with bind silane. If it has been contaminated, soak it in 3 per cent Sodium hydroxide but never soak the two plates (Back plate and notched plate) in the same tray because this causes a cross contamination of both glasses.

1. Select a pair of glass plates to be used. One notched plate and one unnotched gel plate.

2. Clean both the plate using 70 per cent ethanol, wiping it with fur free wipe across and up and down three times.

3. Apply the Bind Silane to the gel plate. For this, mix 1.1 ml 95 per cent Ethanol: 0.5 per cent Acetic Acid solution with 2.0 µl Bind silane in an eppendorf tube, apply the Bind silane solution to the surface of the plate and very quickly, wipe the glass plate as evenly as possible. Let the glass dry for 5 minutes. Change gloves (to avoid contamination).

4. Treat the inner side of notched glass plate with 2-3 sprays of Serva (Blueslick) and wiping the glass plate vertically then horizontally quickly with Kim wipes. Wait 5 minutes to dry.

6. Assemble the glass plate unit lay the back glass plate on the bench with treated face up. Lay the water soaked spacers on both sides of the back glass (gel plate).
7. Carefully lay the notched plate, treated side down facing the gel plate on top of the bottom assembly. Working from the bottom corner, place clamps on both sides of the corner to firmly hold the glass plates together assuring a tight seal. Continue placing clamps on both sides of the glass unit.

8. Stand glass plate unit upright using the clamps as base and lock them with clips. Be sure the grooved surface of the plate faces up.

9. Slowly and carefully pour the acrylamide gel mixture between the glass plates up to the top using 50 ml syringe with no bubbles trapped.

10. Insert the inverted comb if bubbles are trapped, gently lift the comb and push down allowing the bubbles to escape. Wait 1 hour for the gel to polymerize.

11. After the gel has completely polymerized, lay the apparatus on the bench, remove the clamps, and gently remove the comb.

12. Flush out the excess of polymerized gel with mild scratching then removing gel traces by washing the groove with slight pressure from wash bottle. This will prepare uniform gel surface for holding comb.

13. Holding the gel unit from both sides, place the unit carefully in the bottom reservoir which has been filled with approximately 300 ml 0.5 X TBE solution notched plate facing inward. Verify that the upper buffer reservoir is closed and fill the top reservoir with 1X TBE solution to cover the top of the gel. Make sure that there are no buffer leaks from the upper reservoir.

(II) — Gel Loading

1. Warm up gel at 80-90W for 1 hour.

   Note: Another commonly used, and preferred, option is to heat the buffer (2000ml) in the microwave on high power for 5 min.

2. Add 4µl of formamide loading dye to 10µl of reaction solution.

3. Denature the PCR reactions for 5 min at 95ºC or boiling and immediately put into ice.

4. Load 4.5 µl of sample using Hamilton syringe on each lane of 6 per cent non-denaturing PAGE and as the base pair marker, 100bp DNA ladder was loaded
Note: This is a good time to make fix/stop, silver-staining and developing solutions and place them in the freezer (−20°C); the colder the solutions the better the quality of bands.

5. After loading the sample, attach the upper safety cover. Connect the unit to the power supply with proper polarity: black leads connected to the black cathode and the red leads connected to the red anode. Turn on the power pack, set the voltage to 800V, and allow the gel to run for 2.5 hours. Monitor the progress of electrophoresis following the migration of the dye.

6. When the electrophoresis is complete, turn off the power pack and disconnect both power cords from the power supply. Drain the top reservoir by unclamping the binder clip on the clear plastic tube allowing the TBE solution to drain into a beaker. When all of the solution has been drained, remove the top cover by holding the cover.

(III) Silver Staining

1. Separate plates while keeping the gel attached to outer glass plate.

2. Fix the gel by placing gel in tray with cold fix/stop solution (do not pour directly onto the gel) and agitate gently (place on the orbital shaker) for 20 minutes. Gel may be stored in fix/stop solution overnight (however, bands get fatter when you do this). Save fix/stop solution and place back in the freezer.

3. Wash the gel and rinse it three times in 1 litre for 5–10 min each in ddH₂O (double distilled water) using agitation. Lift gel from solution and allow it to draining 10–20 seconds.

4. Staining of the gel is done by pouring the staining solution into the tray. Transfer the gel to staining solution and agitate for 30 min. Remove gel from tray and stand upright. In the last 10 min, add the final reagents to the developing solution.

5. Rinse the gel tray by approx. 1 lit of ddH₂O. Submerge the gel and agitate for 3–7 seconds only. Note: Too much time in the rinse will result in weak staining. Remove the gel from the tray and stand upright.
6. Develop the gel by pouring 1L of developing solution into the tray. Place the gel in the tray and agitate until bands begin to appear.

**Note:** This is a temperature sensitive reaction. The colder the solution the longer it will take for the bands to appear but the clearer they will be; a slushy consistency produces the sharpest bands. Pour in the rest of the chilled developing solution and keep agitating until bands are clearly visible.

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**Note:** Do not forget the gel will continue to get darker until neutralize the developing solution

7. In order to neutralize the gel add 2L of fix/stop solution directly to developing solution and agitate for 2-3 min. (use your hands and pull the plate up and down to more quickly neutralize the developing solution will prevent the gel from getting too dark).

8. Rinse gel twice in 1litre for 2-3 min. in ddH2O using agitation

9. Dry gel on glass by standing the plate upright.

10. After observation on light box or scan of gel, glass plates must be clean up by soaking the gel plate in 3 per cent sodium hydroxide solution. After soaking few hours use a razor blade to scrape the gel from the gel plate.

**Note:** The short glass plate should not be soaked unless it has been contaminated with bind-silane. If it has been contaminated, soak it in 3 per cent Sodium hydroxide but never soak the two plates (Back plate and notched plate) in the same tray because this causes a cross contamination of both glasses.

### 3.5 Bulked segregant analysis (BSA) for identification of putative linked markers

Bulked segregant analysis (Michelmore et al. 1991) was used for the identification of markers linked to the anthracnose resistance locus. Two contrasting DNA bulks, resistant and susceptible, were made by pooling equal amounts of DNA of 20 homozygous resistant and 20 homozygous susceptible F2 individuals of cross Jawala x KRC5. A total of 95 RAPDs and 51 SSR polymorphic on both parents and distributed over the 11 chromosomes of beans were used to screen the two contrasting bulks.

### 3.6 Linkage and mapping analysis
The markers differentiating resistant and susceptible bulks were used for the linkage analysis of an F2 mapping population comprising of 159 susceptible and 40 resistant plants. The linkage distance (d) in cM (Centi Morgan) units between the anthracnose resistance gene using the Kosambi function (Kosambi 1943) was drawn with software MapDisto 1.7.1 (Lorieux 2012).

The SCAR marker ScOPF6522 (forward 5' -GGGAATTCCGGGACAAAACGTGATAAAT-3' and reverse: 5' -GGGAATTCCGTTGGAGATTATTACATGGA-3') linked to R-gene Co-ind (KRC5) in trans phase developed in our laboratory during the previous study was used as marker as per the procedures described in section 3.4.2.

**3.7 Characterization of resistance gene linked region**

The PCR product of Jawala and KRC5 were amplified with OPR15 primer (GGACAACGAG) and run on 1.5 percent agarose gel to obtain a desired target band. The PCR product was cloned and custom sequenced to get the sequence of the amplified region as per the procedure described below.

**3.7.1 Purification of PCR product**

The amplified PCR fragments were column purified using the Qiagen gel extraction kit protocol following the manufacturer’s instructions. Sharp scalpel blade was used to excise the gel slice containing the DNA fragments of interest. The gel slice (about 300 mg) was placed in a sterile 1.5 ml centrifuge tube and 500 μl of DF buffer was added. The contents were incubated at 60°C for 5-10 min until the gel gets completely dissolved with intermittent shaking by inverting after every 1 to 2 min. The gel mixture was allowed to cool down at room temperature after incubation and DF column was placed into a collection tube. About 800 μl of dissolved gel mixture was loaded into the column. Centrifugation was done for 30-60 sec and flow through was discarded. Column was washed once with 400 μl W1 buffer by centrifuging for 30-60 s. Flow through was discarded and 600 μl of wash buffer (ethanol added) was added and kept for 1 min and subjected for centrifugation at 13,000 rpm for 30 s. The flow through was discarded and the column matrix was dried by centrifuging for 3 min at 13,000 rpm. The dried column matrix was transferred in new centrifuge tubes and 50 μl of nuclease free water was
added and allowed to be absorbed by the matrix by keeping for 2 min at room temperature and then centrifuged at full speed to elute the DNA.

3.8 Cloning and sequencing

3.8.1 Preparation of Luria bertani broth and Luria bertani agar plates

In 800 ml of double distilled sterilized water, 25 g of Luria Bertani was added and pH was adjusted to 7.5 with 1N NaOH using pH meter (Cyberscan 2100, Eutech) and then final volume was made to 1000 ml. For the preparation of LB agar plates, 15 g of agar (Merck Ltd.) was added per 1000 ml LB. LB Broth and LB agar media were autoclaved at 15 lbs/inch$^2$ for 15 min in autoclave. LB agar plates were prepared by pouring 25-30 ml of LB agar medium into sterilized disposable petri plates (Axygen) under laminar air flow.

3.8.2 Inoculation of LB agar plates

LB agar plates were inoculated with $DH5\alpha$ strain of bacteria $Escherichia coli$ using streak plate method and then kept for incubation at 37°C overnight.

3.8.3 Inoculation of LB broth

A single bacterial colony from overnight grown culture was taken to inoculate 5 ml of LB broth in a 15 ml snap cap tube. After inoculation the culture tube was incubated overnight at 37°C in a shaking incubator.

3.8.4 Preparation of competent cells

250 ml LB broth was inoculated with 1 ml of overnight grown bacterial culture of $E. coli$ strain $DH5\alpha$ and incubated at 37°C in a shaking incubator till OD reaches at 0.5 at 600 nm (approximately 2 hours) measured using spectrophotometer (Biorad). The culture was cooled on ice for 15 minutes and centrifuged at 6000 rpm for 15 min at 4°C. Supernatant was discarded carefully and the bacterial pellet was resuspended in 50-70 ml ice cold 0.1M CaCl$_2$ and incubated once for 30 min. The cells were collected by centrifugation at 6000 rpm for 5 min at 4°C and bacterial pellet was resuspended in 50-70 ml ice cold 0.1M MgCl$_2$ and again incubated on ice for 30 min. Cell suspension was again subjected to centrifugation at 6000 rpm for 5 min at 4°C. Pellet was finally resuspended in 10 ml of 0.1M CaCl$_2$ containing 10 per cent glycerol. 200 μl aliquots of
competent cells were prepared in sterilized 1.5 ml microfuge Eppendorf tubes and stored at -80°C.

3.8.5 Ligation of eluted DNA into vector

The eluted PCR products representing the various genome segment were ligated in pGEMT- Easy vector (Promega Corp., Madison, WI) according to the manufacturer’s instructions using 10 μl reaction volume containing 5 μl 2X rapid ligation buffer, 0.5 μl pGEMT-Easy vector, 3 μl template (eluted DNA), 1 μl T4 DNA ligase and finally nuclease free water was added to make the final volume. The reaction volume was vortexed and centrifuged briefly and incubated overnight at 4°C.

Fig 3.3. pGEM-T Easy vector (Promega Corp., Madison) used for ligation of eluted PCR product

3.9.6 Transformation of competent cells of E. coli strain DH5α with ligated product

Frozen competent cells stored at -80°C were allowed for thawing on ice for 10-15 min. 10 μl of ligated product was added to the tube containing competent cells, mixed well by tapping and incubated on ice for 30 min. Heat shock was given to the cells at 42°C for 90 s in water bath and immediately transferred to ice for 15 min. 800 μl of fresh sterilized LB broth without antibiotic was added to this and the tube contents were incubated at 37°C for 1 h in an incubator shaker (150 rpm). Cells were subjected to
centrifugation at 12,000 rpm for 30s after incubation. Top 900 µl of the supernatant was discarded while remaining 100 µl of supernatant was mixed thoroughly with the pellet formed by pipetting and spreader on LB agar plates containing Ampicillin, IPTG and X-gal. The plates were then sealed with parafilm and incubated overnight (18 h) at 37°C.

3.9.7 Colony PCR check

After overnight incubation, randomly 10 white colonies were picked up and replica plates were made with the sterilized micropipette tip and the same tip was dipped into 5 µl nuclease free water in eppendorf tube for the confirmation of transformation. Replica plates were incubated overnight at 37°C and then stored at 4°C. Eppendorf tubes containing colony in nuclease free water was kept for 7 min in boiling water bath and immediately kept on ice. The colony PCR was performed in 10 µl reaction volume containing 1.0 µl 10 X PCR buffer, 0.5 µl 25 mM MgCl₂, 0.6 µl 2.5 mM dNTPs, 0.4 µl each 10 µM forward and reverse primer, 0.2 µl 5U/µl Taq Polymerase, 2.0 µl colony, nuclease free water was added to adjust the final volume. Amplification was performed in GeneAmp PCR system 9700 (Applied Biosystems) with initial denaturation of 94°C for 4 min followed by 30 cycles of 94°C for 15 s, annealing at 48°C for 40s and extension at 72°C for 1 min with a final extension of 5 min at 72°C.

3.9.8 Plasmid isolation by alkali lysis method

Test tubes containing 10 ml of LB containing the antibiotic was inoculated with a single isolated colony picked from an LB agar plate containing Ampicillin, IPTG and X-gal and incubated overnight (18 h) at 37°C in a rotary shaker. 1.5 ml of the overnight grown culture taken in a microfuge tube subjected to centrifugation for 1 min at 14,000 rpm to pellet down the cells. Supernatant was discarded and previous step was repeated. Bacterial pellet was resuspended in 100 μl of ice cold alkali lysis solution I by pipetting or by vortexing. Two hundred μl of freshly prepared alkali solution II and 4 μl of RNase (10 mg/ml) were added to each bacterial suspension. Contents were mixed by inverting the tubes and stored at room temperature for 10 min. To it 150 μl of alkali lyses solution III was added and the contents were mixed. The content was stored on ice for 5 min and after that lysate was subjected to centrifugation at 14000 rpm for 10 min at 4°C. Aqueous phase was transferred to fresh tubes and nucleic acid was precipitated from supernatant by adding 400 μl of chilled isopropyl alcohol. Contents were mixed by vortexing and
centrifuged at 14000 rpm at 4°C for 15 min. Supernatant was discarded and tubes were kept inverted on paper towel to drain out the excess fluid. 1 ml of 70 per cent ethanol was added and washing was done by centrifugation at 14000 rpm for 5 min at 4°C. Supernatant was discarded and tubes were dried until ethanol evaporated. Pellet was dissolved in 50 μl of sterilized RNase free water. Purified Plasmid DNA was checked by agarose gel electrophoresis using 3 μl of DNA mixed with 2 μl of 6X gel loading dye.

45 μl of purified plasmid DNA containing genome fragments were freeze dried in lyophilizer (Alpha 1-2 LD) and submitted for custom sequencing (Xcelris Labs Ltd). Sequencing of plasmid was carried out by using universal primers (SP6 and T7) in both the directions by using Chain-termination (Dideoxy) method.

3.10 Physical delineation of anthracnose resistance locus and identification of candidate resistance gene(s)

Nucleotide sequences obtained after custom sequencing were first screened for vector sequences using vecscreen software. The physical region spanning the anthracnose resistance locus was delineated by landing the sequences of flanking markers on the sequence of Andean pool landrace line (G19833) genome using BLAST option search in webpage (http://www.phytozome.net/common bean). Candidate resistance gene(s) were identified by searching the equivalent genomic region of Andean genotype G19333 for NBS-LRR genes using the Phytomine tool (http://phytozome.jgi.doe.gov/phytomine/begin.do).

3.11 Evaluation of host resistance and validation of markers

A set of one hundred seventy eight indigenous and exotic lines of common beans were evaluated for resistance gene against race 3(Cl-186a) of *C. lindemuthianum* by seedling dip method (Champion et al. 1973) under controlled conditions Section 3.2.4. DNA of highly resistant lines exhibiting 0 or 1 reaction was tested for presence of resistance gene using molecular marker using linked marker viz., OPR15136, OPF6522, SQ4, SAS13, SBB14, SC08, SY20, SAB3, SZ20 and SF10.

Table 3.4. List of tested SCAR markers linked with disease resistance traits in common bean (*P. vulgaris*)

<table>
<thead>
<tr>
<th>SCAR Name</th>
<th>Marker of Origin</th>
<th>Pathogen</th>
<th>Size (bp)/ orientation</th>
<th>Sequences of SCARS</th>
<th>Tagged Locus</th>
<th>LG</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ4</td>
<td>OQ4</td>
<td>Anthracnose</td>
<td>1440</td>
<td>CCT TAG GTA TOG TOG GAA ACG A</td>
<td>Co-2, 11</td>
<td></td>
<td>Awale et al. 2008;</td>
</tr>
<tr>
<td>Accession</td>
<td>Strain</td>
<td>Pathology</td>
<td>Position</td>
<td>Allele</td>
<td>SNP</td>
<td>Genotype</td>
<td>Reference</td>
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<tr>
<td>-----------</td>
<td>--------</td>
<td>-----------</td>
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<tr>
<td>SAS13</td>
<td>AS13</td>
<td>Anthracnose</td>
<td>950</td>
<td>cis</td>
<td>CAC GGA CCG AAT AAG CCA ACA</td>
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<td>8</td>
</tr>
<tr>
<td>SBB14</td>
<td>BB14</td>
<td>Anthracnose</td>
<td>1150/1050</td>
<td>codominant</td>
<td>GTG GGA CCT GTT CAA GAA TAA TACGTG GGA CCT GGG TAG TGT AGA AAT</td>
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<td>8</td>
</tr>
<tr>
<td>SY20</td>
<td>Y20</td>
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<td>830</td>
<td>cis</td>
<td>AGC CTT GGA AGG TTG TCA T</td>
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<td>8</td>
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<tr>
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<td>C08</td>
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<td>cis</td>
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<td>7</td>
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<tr>
<td>SF10</td>
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<td>Anthracnose</td>
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<td>cis</td>
<td>GGA AGC TTG GTG AGC AAG GA</td>
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<td>4</td>
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