3. Material and Methods

The purpose of the study was achieved in following steps:

1. Phenotypic assessments of barley accessions of both indigenous and exotic nature for leaf blight infection in field under epiphytotic conditions.
2. Genotyping of barley accessions using molecular markers reported for QTL regions of leaf blight resistance to find closely linked markers associated with leaf blight resistance.
3. Selection of Barley lines used as parental lines to develop RILs for the study of leaf blight inheritance.
4. Extensive phenotypic screening of the RILs (Recombinant Inbreed Lines) developed from cross DWR49 X RD2503 along with the Parents for leaf blight infection in field under epiphytotic conditions.
5. Genotyping of the RILs of cross DWR49 X RD2503 using BSA (Bulk Segregant Assay) after parental screening.
6. To study the inheritance of leaf blight resistance in Indian barley.
7. Identification of molecular markers closely associated with leaf blight resistance in Indian barley lines.
8. Identification of QTLs (Quantitative Trait Loci) associated with leaf blight resistance in Indian barley lines.

3.1 Material

3.1.1 Plant material

A: Germplasm Accessions

A set of 85 barley germplasm accessions was used to study leaf blight resistance in this study. These genotypes were procured from Barley Network Section, Directorate of Wheat Research, Karnal. These barley lines were of both indigenous and exotic nature (Table 3.1)
### Table 3.1 Barley accessions (Indigenous and exotic) used for leaf blight resistance study during three consecutive years (2004-07)

<table>
<thead>
<tr>
<th>Origin</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigenous</td>
<td>KARAN-757, KARAN-1057, HBL 233, VLB 35, Line 1243-3, IC-25615,</td>
</tr>
<tr>
<td></td>
<td>IC-36899, IC-58028, IC-61844, IC-62765, IC-79593, IC-437886, IC-437955,</td>
</tr>
<tr>
<td></td>
<td>IC-437956, IC-437958, IC-437959, IC-437960, IC-437996, IC-438160, IC-</td>
</tr>
<tr>
<td>Exotic</td>
<td>BON-LRA-M(90-91)-86, BON-LRA-M(90-91)-88, 19th IBON (91-92)-2819th</td>
</tr>
<tr>
<td></td>
<td>IBON (91-92)-86, PUBEBLA, 2nd IWFBON-18, 2nd IWFBON-23, 2nd IWFBON-98,</td>
</tr>
<tr>
<td></td>
<td>2nd IWFBON-103, 2nd IWFBON-114, 2nd IWFBON-117, BON-LRA(91-92)-12, 1st</td>
</tr>
<tr>
<td></td>
<td>HBSN 1, BON-MRA(91-92)-93, TOKAK, ICB-94(QUINN/BC//ATHS/ANTARES), ICB-</td>
</tr>
<tr>
<td></td>
<td>13(WI22914/AVT/KI//AVT/3/TOLI/BZ), 20th IBYT 10, 20th IBYT-11, 21st IBYT-</td>
</tr>
<tr>
<td></td>
<td>7, 21st IBYT-10, 21st IBYT-14, 21st IBYT-17, 21st IBYT-18, 27th IBON-8,</td>
</tr>
<tr>
<td></td>
<td>27th IBON-9, ISBCB-82(ALANDA-01/3/EMIR//ESP/SV. MARI ICB89-0779-4AP-OTR-</td>
</tr>
<tr>
<td></td>
<td>3AP-OTR, 29th IBON 3, 29th IBON 9, 29th IBON 11, 29th IBON 13, 29th IBON</td>
</tr>
<tr>
<td></td>
<td>20, 29th IBON27, 29th IBON 41, 29th IBON 43, 29th IBON 44, 29th IBON 49,</td>
</tr>
<tr>
<td></td>
<td>29th IBON 65, 29th IBON 70, 29th IBON 75, 29th IBON 170, 10th EMBSN 5,</td>
</tr>
<tr>
<td></td>
<td>10th EMBSN 8, 10th EMBSN 20, 10th EMBSN 29, EC-492144, EC-492147, EC-</td>
</tr>
<tr>
<td></td>
<td>492152, EC-492193, EC-492196, EC-492198, EC-492220, EC-492222, EC-492229,</td>
</tr>
<tr>
<td></td>
<td>EC-492238, EC-492254, EC-492285, SEVIROWS(B), Keel, Schooner.</td>
</tr>
</tbody>
</table>
**B: Recombinant Inbred Line Population:** The RIL population (F₈) of cross DWR49 and RD2503, developed for leaf blight resistance studies under molecular markers assisted barley improvement research project at Barley Network Section, Directorate of Wheat Research, Karnal was used in this study. RILs population was developed by crossing two rowed resistant line, DWR49 with six rowed susceptible line, RD2503.

DWR-49, which shows a high level of resistance to leaf blight originated from breeding programs at DWR, Karnal. It is a genetic stock registered with NBPGGR, New Delhi for spot blotch resistance in barley. It showed resistance up to 13 scales that is considered as the best observation for leaf blight in NWPZ and NEPZ regions. RD2503 is a high yielding and best Indian malt variety among 6 rowed barley lines but it is highly susceptible to leaf blight. 142 RILs (Recombinant Inbreed Lines) of F₈ generation developed from cross DWR49 X RD2503 were used for genotyping. The seed materials were procured from Barley Section of Directorate of Wheat Research (DWR), Karnal.

**3.1.2 Chemicals**
Chemicals for molecular work were purchased from Himedia Chemicals Co. USA and SRL, India. Taq DNA polymerase, dNTPs for polymerase reaction and ladder to compare the band size of amplified DNA fragment were purchased from New England Biolabs (NEB) and Genei, Bangalore. Rests of the chemicals for the investigation were of molecular biology grade or analytical grade and were procured from Sigma Chemicals Co., USA; Promega Inc., USA; Gibco BRL Inc., USA and E. Merck, India. Different reagents used in molecular study are given in Annexure 1.

**3.1.3 Molecular markers**
The random or specific molecular markers covering all the seven chromosomes of Barley were used in this study. Primer sequences for SSR markers were obtained from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml). The microsatellite markers of Bmag, Bmac, EBmac, HVM and GBM series, specific STS markers and EST based SSR markers were used to screen plant material. These markers were got synthesized from Sigma Aldrich Chemicals, India. Molecular markers used for study of leaf blight resistance in RIL population and germplasm accessions are listed in table 3.2 and 3.3.
Table 3.2 Molecular markers (identified for genomic regions associated with leaf blight resistance) used for screening of barley genotypes

<table>
<thead>
<tr>
<th>Chromosome location</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>Bmag504, Hor2, HVM20, HVCMA</td>
</tr>
<tr>
<td>2H</td>
<td>ABG397, Bmac93, EBmac623, EBmac684, EBmac715, GBM1024, MWG64</td>
</tr>
<tr>
<td>3H</td>
<td>Bmag013, Bmag225, Bmag518, Bmag606, Bmag905, Bmag919, EBmac705, GBM1034, GBM1073, GBM1159, HvLTTP</td>
</tr>
<tr>
<td>5H</td>
<td>BMS32, BMS90, Bmag09, HvLeu</td>
</tr>
<tr>
<td>7H</td>
<td>ABC 167A, ABG380, Bmag135, EBmac603, EBmag794, HVM4, Ris44</td>
</tr>
</tbody>
</table>
Table 3.3 Molecular markers screened for leaf blight resistance with parents (DWR49 & RD2503)

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>Ksud14, Mwg835a, Mwg938, Abc165c, Bmac399, Aba004, Bmac213, Abg059, Ebmac405, Bmag211, Bmag105, Bmac154, Scssr10477, Hvhva1, Abg702a, Abc322b, Abc261, Gbm1204, Mwg2021, Abc257, Abc322b, Abg500a, Bcd304, Bmag382, Bmag589, Bmag872, Mwg912, Ebmac501</td>
</tr>
<tr>
<td>4H</td>
<td>Mwg634, Mwg2282, Abg313b, Wg622, Gbm1221, Hvm40, Bmag106, Hvole, Scssr20569, Bmag740, Hvkn0x3, Gbm145, Bmag740, Bmac310, Hvm13, Bmag353, Scssr14079, Gbm1299, Bcd453b, Ebmac701, Hvmloh1a, Ksug10, Ebmac635, Abg500b, Hvm67, Gbm1015, Abg319c, Gbm1388, Bmy1, Hdanyb, EBMAC906, Ebmag781, Gbm1448, Gbm1388, Gbm1299</td>
</tr>
<tr>
<td>5H</td>
<td>Sccsr02306, Abc483, Abc717, Abg705a, Bmag337, Abc164b, Bmac113, Hveu, Bmag337, Abc302, Bmag760, Ebmac854, Hvbamy, Ksua1, Bmag812, Scind16991, Gbm1438, Abg702b, Ebmac824, Ebmatc003, Gbms141, Abg712, Gms27, Abg391, Abg463, Sccsr03907, Mwg851, Mwg851b, Abc254, Mwg9201, Abmag387, Bmag223, Hvdhn09, Gms61, Bmag222, Gms1, Gbm1229, Gbm1506, Bmag337</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6H</td>
<td>Abg62, Abg378, Bmag500, Ksua3b, Abg387b, Gbm1215, Ebmac9706h, Hvm14, Ebmac874, Hvlox, Ebmac806, Mwg8206h, Hvm11, Sccsr05599, Mwg934, Sccsr00103, Abc170a, Abc154, Gbm1275, Ksuf37, Mwg798a, Bmag173, Gbm1423, Gbm5012, Bmac0040b, Mwg934, Mwg934, Gbm1274, Gbm1087, Gbm1404, Bmag798a, Gbm1423, Gbm5012, Bmac0040b, Bmag0103a, Gbm1356, Mwg934, Gbm1274, Gbm1087, Gbm1404</td>
</tr>
<tr>
<td>7H</td>
<td>Abg704, Ebmac713, Bmag206, Ebmag794, Abg380, Abc167a, Abc158, Mwg836, Sccsr7970, Brz, Hvcma, Bmac187, Mwg2271, Abg701, Bmag110, Gbm1492, Bmag183, Bmag217, Bmag516, Bmac162, Bmac273, Bmac47d, Bmac31, Bmag011, Bmag582, Bmag369, Bmag571a, Bmag746, Bmac31, Bmac64, Abc310, Bmac579, Abg476, Awbms22, Ris44, Abc253, Hvm49, Bmag135, Gbms183, Hvplasc1b, Sccsr15864, Mwg911b, Mwg2304, Ebmac827, Bmac224, Mwg808, Bmag341, Bmag369, Bmac167</td>
</tr>
</tbody>
</table>
3.2 Techniques used

3.2.1 Genomic DNA extraction

Genomic DNA was isolated from the fresh leaves of 14 days old seedlings using modified CTAB method in bulk or by miniprep DNA isolation method (Saghai-Maroof et al. 1984) as explained below. DNA was extracted from all 85 barley accessions and also from the Parental lines DWR 49 & RD2503 and RIL population (F₈ generation) of cross DWR 49 X RD2503.

a) Bulk DNA extraction

1. Young leaf samples from 2-3 week old seedlings taken and grinded into fine powder using liquid nitrogen in a sterile pestle and mortar.

2. Approximately five gram of the grinded leaf tissue powder was mixed with 15 ml of preheated (65°C) CTAB buffer and incubated at 65°C for 90 minutes with regular gentle mixing of samples.

3. After incubation, samples were cooled to room temperature and 15 ml of Chloroform: Isoamylalcohol (24:1) mixture was added and mixed well by inverting the tubes gently for 10-15 min.

4. Tubes were then centrifuged at 10,000 rpm for 10 min. and upper aqueous layer was mixed with equal volume of ice-cold isopropanol to precipitate DNA.

5. The DNA was then spooled out and centrifuged at 12,000 rpm for 5 min to pellet down. The DNA pellet was given 70% ethanol washing and left overnight for drying. DNA was subsequently dissolved in appropriate volume of TE buffer and samples were stored at -20°C till further use.

RNase treatment

RNase treatment was given for DNA purification to remove RNA impurity.

1. Add DNAse free RNAse (30mg/100ml DNA) to the dissolved DNA sample.

2. Incubated for 30 min at 37°C.

3. DNA was extracted with Chloroform: Isoamylalcohol (as DNA isolation in step 3).
4. It was centrifuged at 10,000 rpm for 10 min.

5. About 1/10\textsuperscript{th} volume of 3M Sodium acetate (pH 6.8) and 2 volumes of 95% ethanol was added.

6. Pellet down the precipitated DNA and dissolved it in TE buffer after drying overnight in air.

b) Miniprep genomic DNA Isolation

1. 2-3 fresh leaves were grinded in liquid nitrogen in the micro centrifuge tube.

2. 700 µl of preheated (65°C) CTAB-DNA extraction buffer was added and mixed well.

3. Incubated for one hour at 65°C with gentle shaking after every 10 min by inverting the tubes.

4. The tubes were removed from the water bath and cooled. 700 µl of Chloroform: Isoamylalcohol (24:1 v/v) was added and gently mixed and centrifuged at 12,000 rpm for 10 minutes.

5. Transferred the supernatant to a fresh 1.5ml microcentrifuge tube and added 4 µl of RNAse and incubated at 37°C for 30min

6. 25 µl of sodium acetate was added and the tubes were incubated for 1 min.

7. 800 µl of ice-cold Isopropanol was added and the tubes were kept undisturbed for 15 min.

8. The DNA was pelleted down by centrifugation at 12,000 rpm for 10min.

9. Added 300 µl of 70% ethanol and centrifuged at 8000rpm for 1-2 min.

10. The DNA was kept for air-drying overnight. Dissolved it in minimum amount of TE buffer and stored at -20\textdegree C for further use.

3.2.2 PCR analysis

PCR amplifications were carried out at optimum annealing temperatures for respective primers to perform parental screening, BSA and genotyping of RILs. PCR is an \textit{in vitro} procedure for
the enzymatic synthesis of DNA, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. The procedure enables small amounts of specific DNA fragments to be amplified between $10^6$ and $10^{12}$ times.

PCR for the amplification of template DNA was done using Gradient Thermocycler PTC-100™ (Biorad, USA). Total volume of PCR reaction mixture was kept 25 µl containing 1 x PCR buffer, 200 µM dNTPs, 300 µM of primer (both), 1.5 mM MgCl$_2$, 1 unit Taq DNA polymerase and 40-50 ng template DNA. PCR amplification was performed as per reported PCR conditions for individual molecular marker (www.graingenes.com).

General PCR reaction followed while performing PCR amplifications:

1. Initial denaturation  
   $94^\circ C$ for 3 min.
2. Denaturation  
   $94^\circ C$ for 1 min.
3. Annealing  
   $60^\circ C$ for 1 min.
4. Extension  
   $72^\circ C$ for 2 min.
5. Final extension  
   $72^\circ C$ for 10 min.

Cycles were set by repeating steps 2 to 4 for 35 times and amplified products were stored finally at -20°C till further use.

3.2.3 Agarose gel electrophoresis

PCR products were resolved on 1.5% (w/v) agarose gel by submerged horizontal electrophoresis. Washed and dried gel casting plate was sealed at its both ends with tape. Agarose was melted in 1 x TBE, ethidium bromide was added at concentration of 5 µg/ml and poured into plate having comb to get 0.5 cm thickness of gel. Sealing tapes were removed after polymerization of agarose and gel casting plate was merged in electrophoretic chamber containing 1 x TBE. 6 x loading dye was added to the DNA samples and the samples were loaded in the wells and electrophoresis was done at constant voltage (3v/cm of gel). Bands were viewed under UV light after electrophoresis and saved in gel documentation system.
3.3 Methodology

3.3.1 Phenotypic screening for leaf blight resistance

Phenotypic screening was conducted at experimental fields of Directorate of Wheat Research (DWR), Karnal during crop seasons 2010 - 2012. All the selected barley lines (RILs of cross DWR49 X RD2503 and germplasm accessions) were grown in the field during crop season (2010 to 2012) with the highly susceptible variety RD2503, used as infector at DWR, Karnal under epiphytotic conditions. The infector was grown at right angle to the direction of test lines every year. The field inoculation was started with multiplied inoculum in mid of January and continued till first week of March. The inoculum of *Bipolaris sorokiniana* was obtained from Plant protection section, DWR, Karnal. The inoculum was prepared by inoculating infected barley leaves on Potato dextrose Agar Media (PDA). The inoculum was preserved on sorghum grain and for multiplication the extracted culture was suspended in sterile distilled water and store at 4-5°C till reaches the appropriate growth stage. The spores of fungal pathogen were harvested in water. A spore suspension (approximately $10^4$ spore/ml) containing surfactant Tween 20 was uniformly sprayed by using a hand held atomizer at three stages: tillering, flag leaf emergence and anthesis during the evening hours (Joshi et al., 2007b, c).

First symptoms were recorded around first week of March on the infector (RD2503), which spread further in size on the leaves and to adjoining plants/rows in about next 15 days. The disease peaked around end of the March. Three recordings were taken at six days intervals for every crop season starting in mid of March. The data recorded on these genotypes in first two observations was recorded in double digit system indicating the % area covered on the flag leaf and on the next below to flag leaf on 1 to 9 scale (Kumar et al. 1998; Nagarajan and Kumar 1998). The third observation was recorded on overall reaction of the plant on 1-9 scale for classification (Fletch and Steffenson, 1999) and plants were categorised as resistant (up to 3), moderately resistant (3-5), moderately susceptible (5-7) and susceptible (>7).

3.3.2 Molecular screening for leaf blight resistance

A) Molecular screening of barley accessions: 33 molecular markers available from reported closely linked for QTL regions of leaf blight resistance in earlier studies were used to screen 85 barley accessions for their applicability in selected barley lines (Table 3.2).
B) Molecular screening of RILs of DWR49 X RD2503:

Parental screening

The random and specific molecular markers covering all the seven chromosomes of Barley were used in this study. 283 microsatellite markers of Bmag, Bmac, EBmac, HVM series, specific STS markers and EST based SSR markers were used to screen parental lines (Table 3.3).

Genotyping of RIL population

Resistant bulk and susceptible bulk were made by pooling together DNA of ten most resistant and ten most susceptible RILs respectively. The markers found polymorphic on the parental lines during initial screening were further screened with these two bulks of RILs. All the markers which were found polymorphic on bulks of RILs were used for genotyping on 142 RILs along with the parents.

3.4 Linkage analysis

Only clear and apparently unambiguous bands were scored. The size of the most intensely amplified band was determined based on its migration relative to molecular weight size ladder.

3.4.1 Chi- square analysis

The observed and expected distribution of the RILs for leaf blight resistance were tested using chi-square ($\chi^2$) analysis to study the inheritance pattern of markers associated with leaf blight resistance. Genetic hypothesis was tested by Chi square ($\chi^2$) test analysis, to check the validity of expected ratio in the mapping population for analyzing the inheritance of leaf blight resistance in association with molecular markers. Leaf blight resistance was determined by range of the parents and with rejection at the 0.01 level of probability.

3.4.2 QTL analysis

Composite interval mapping was undertaken using Windows QTL Cartographer version 2.5 (Wang et al., 2001-2004), employing model 6 with a 10 cm window and 2 cm walk speed. One thousand (1000) permutation tests at 2 cM intervals were conducted to determine significance thresholds for QTL detection. The marker allele $sr$ (spot blotch resistant allele) was coded 2 and the allele $ss$ (spot blotch susceptible allele) were coded 0 for conducting regression
analysis. Genetic linkage analysis for SSRs was performed using reference map (Varshney et al., 2007) with a LOD score of 3.0. Recombination frequencies were transformed to centiMorgans (cM) using the formula of Kosambi.