3.1 Genotypes

The genotypes used in the present study for the crossing programme were as follows:

(a) *B. juncea* parents: Three new *B. juncea* brown seeded genotypes, RESBJ 837 (Accession No. EC 446033), RESBJ 830 (Accession No. EC 446032) and TERI (OE) M21-1 were used as female parents. Among the three, RESBJ 837 and RESBJ 830 (obtained courtesy Dr N I Nashaat, Rothamsted Research, Harpenden, UK) were selected as F₄ generation of a cross involving selected S₃ lines from var. Kranti and var. Krishna, respectively. Both genotypes possessed resistance to a wide range of Indian and UK isolates of *P. parasitica* and also showed a differential resistance response to *A. candida* isolates derived from *B. juncea* and *B. rapa* at the cotyledonary leaf growth stage under controlled conditions at Rothamsted Research Station, UK (Table 3.1). These two parent genotypes were moderately susceptible to alternaria blight infection under field conditions (unpublished data). The third *B. juncea* genotype, TERI (OE) M21-1 was a brown seeded near- isogenic line of TERI (OE) M21 (INGR: 98001; yellow seeded) having low erucic acid but susceptible to both white rust and alternaria blight infection under field conditions.

(b) *B. rapa* parents: Two new *B. rapa* genotypes, RESBR 219 (Accession No. EC 446034) and RESBR 350 (Accession No. EC 446035) were used as male donors. These two genotypes (obtained courtesy Dr N I Nashaat, Rothamsted Research, Harpenden, UK) were S₃ progeny of a line selected from var. PYSLP-2 and var. YST-151, respectively. Both genotypes possessed resistance to a wide range of Indian and UK isolates of *P. parasitica* and also showed a differential resistance response to *A. candida* isolates derived from *B. juncea* and *B. rapa* at the cotyledonary leaf growth stage under controlled conditions at Rothamsted Research Station, UK (Dr. NI Nashaat, Personal communication; Table 3.1).

(c) *B. carinata* parent: *B. carinata* var. Kiran (obtained courtesy Prof. S J Kolte, GB Pant University of Agriculture and Technology, India) was used as a male donor. Kiran is resistant to alternaria blight and tolerant to white rust (Prof. SJ Kolte, Personal communication).
Table 3.1 Response of parent lines of *B. juncea* and *B. rapa* to isolates of *Albugo candida* derived from *B. juncea* and *B. rapa*

<table>
<thead>
<tr>
<th>Genotypes</th>
<th><em>B. juncea</em> isolates</th>
<th><em>B. rapa</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2A (25%)</td>
<td>2V (11.11%)</td>
</tr>
<tr>
<td>RESBJ 830</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RESBJ 837</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (45.45%)</td>
</tr>
<tr>
<td>RESBR 219</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- (26.67%)</td>
</tr>
<tr>
<td></td>
<td>+ (73.33%)</td>
<td>+ (7.69%)</td>
</tr>
<tr>
<td>RESBR 350</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- (71.43%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (28.57%)</td>
</tr>
</tbody>
</table>

‘+’ = Susceptible; ‘-’ = Resistant  (Source: Nashaat and Kumar, 1999)

### 3.1.1 Field layout

The fields were prepared as per standard agricultural practices (Reddi and Reddy, 1980). The seeds of parent genotypes, hybrids and their subsequent progenies were grown during four consecutive Rabi crop seasons (October 2001 to April 2005) at the TERI field station, Gual Pahari. All plant progenies were raised in the field as Single Plant Progeny (SPP) rows with 30 cm row-to-row and 10 cm plant-to-plant spacing in the second fortnight of October. The hybrids and their advanced plant progenies were sown in blocks containing progenies, their parent genotypes and infector rows repeated after every 10-12 lines. Two popularly grown Indian varieties, *B. juncea* var. Varuna and *B. rapa* var. Pusa Gold were sown as infector rows along with the plant progenies of cross *B. juncea* × *B. rapa*, while *juncea* var. Varuna was used as infector rows with the plant progenies obtained from cross *B. juncea* × *B. carinata*.

### 3.1.2 Crossing programme

*B. juncea* genotypes were used as female parents, while *B. rapa* and *B. carinata* genotypes were used as male donors for white rust, and both white rust and alternaria blight resistance, respectively. Following interspecific crosses were undertaken for incorporating resistance to white rust and alternaria blight:

I. *B. juncea* × *B. rapa* for white rust resistance
   i. RESBJ 830 (J0) × RESBR 219 (R2): J0R2
   ii. RESBJ 830 (J0) × RESBR 350 (R3): J0R3
   iii. RESBJ 837 (J7) × RESBR 219 (R2): J7R2
   iv. RESBJ 837 (J7) × RESBR 350 (R3): J7R3

II. *B. juncea* × *B. carinata* for white rust and alternaria blight resistance
   i. RESBJ 830 (J0) × Kiran (K): J0K
   ii. RESBJ 837 (J7) × Kiran (K): J7K
   iii. TERI (OE) M21-1 (M21-1) × Kiran (K): M21-1K
3.2 Hybridization

Unopened flower buds of the female *B. juncea* parent genotypes were emasculated one day prior to anthesis and bagged with butter paper bags. Emasculated buds were pollinated next day using pollen from freshly dehisced anthers of the male donors and rebagged. Pollinated pistils were utilized to study the compatibility between the parents through pollen pistil interaction and for generation of hybrids.

3.2.1 Compatibility study

The compatibility between the parent genotypes was studied through pollen pistil interaction following the aniline blue florescence method of Shivanna and Rangaswamy (1992). Four to five pollinated pistils were fixed in acetic-alcohol (Glacial acetic acid and absolute alcohol (v/v) in a ratio of 1:3, respectively) for 24-48 h and then stored in 70% (v/v) ethanol at room temperature, until used. For slide preparation, the fixed pistils were soaked overnight in 4 N NaOH, rinsed in water and mounted in a 1:1 v/v mixture of 0.005% decolourised aniline blue (Appendix I) and glycerine. The pistils were covered with a cover slip and a gentle pressure was applied to spread the tissue evenly. The slides thus prepared were observed under fluorescence microscope (Appendix II) using UV excitation range with barrier filter L 420 at 405 nm for pollen germination and pollen tube growth at the stigmatic surface, stylar and ovular region.

3.2.2 Generation of hybrids

Hybrids were generated using *in vitro* embryo rescue technique and *in vivo* seed set. For this, out of the total ovaries pollinated, 75% pollinated pistils of each cross were utilized for *in vitro* embryo rescue in the form of ovary, ovule and sequential embryo rescue, while 25% were left on plants till maturity to study seed set *in vivo*. The pods left *in vivo* were harvested at maturity.

3.2.2a In vitro embryo rescue

The methodology followed, for the three *in vitro* techniques; ovary culture, ovule culture and sequential embryo rescue to obtain hybrids, is as follows:

For ovary culture, the pollinated pistils were excised 4-6 DAP and surface sterilized using 70% alcohol for 1 min followed by 0.05 % (w/v) mercuric chloride treatment for 7-8 min. After rinsing thrice with sterile distilled water (DW), the pistils were cultured on MS medium (Murashige and Skoog, 1962; Appendix III) supplemented with growth hormones. Medium E1 suggested by Agnihotri (1993) was used for culturing pistils with slight modification in the
composition of growth additives; 1.0 mg/ L Kinetin (Kn), 0.1 mg/ L Naphthalene acetic acid (NAA), 1.0 mg/ L Gibberellic Acid (GA3) and 50 mg/ L Casein hydrolysate (CH) (medium ME1). The swollen pistils were sub cultured on fresh medium ME1 after 2- 3 weeks and dissected after 4- 5 weeks of initial culture. The excised ovules were cultured on fresh ME1 medium and allowed to grow.

For ovule culture, the pollinated pistils were taken 15- 20 DAP, surface sterilized as mentioned above, and dissected. The developing ovules were excised and cultured on fresh medium ME1 and allowed to grow.

In case of sequential embryo rescue, pollinated pistils (4- 6 DAP) were surface sterilized as mentioned above and cultured on medium ME1. The cultured ovaries were dissected after two weeks and the excised ovules were cultured on fresh ME1 medium.

The cultured ovules, obtained from either of the above-mentioned ovary/ ovule culture/ embryo rescue, either germinated directly or were dissected after a week and the developing embryos were cultured on medium ME1 with 1/ 10th concentration of growth regulators (medium ME2; 0.1 mg/ L Kn, 0.01 mg/ L NAA, 0.1 mg/ L GA3 and 50 mg/ L CH). After 2- 3 weeks, the regenerating embryos were transferred to MS liquid medium without any growth regulators and incubated for 24- 48 h on a gyratory shaker at 70 rpm. The embryos were then transferred to solidified MS medium without any growth regulators and allowed to grow into plantlets by sub culturing every 2- 3 weeks.

All in vitro cultures were maintained at 22 ± 2°C at 16 h photoperiod with a photon flux of 170 µ mol m⁻² s⁻¹ throughout the experiments.

### 3.2.3 Raising of hybrids

The putative hybrid plantlets obtained through in vitro embryo rescue were multiplied using axillary bud proliferation and apical meristem culture on MS medium supplemented with 1.0 mg/ L Kn and 3.0 % sucrose, medium M1. The plantlets were sub cultured every 2-3 weeks. The 3- 4 leaf growth stage plantlets were further utilized for rooting. Three rooting mediums i.e. MS + 0.1 mg/ L IBA + 2% (w/ v) sucrose (medium R1), MS + 0.1 mg/ L NAA + 2% (w/ v) sucrose (medium R2) and MS + 1% (w/ v) sucrose (medium R3) were used. To study the efficiency of these three rooting mediums, 24 in vitro raised plantlets were cultured in each medium for 3- 4 weeks. The percent efficiency was calculated as number of plantlets rooted out of total number of plantlets cultured in each medium.

The seeds obtained in vivo were divided into two equal lots. One sown directly in
soil in the field along with parent lines, and the other by germinating on MS medium containing 1% (w/ v) sucrose for 2- 3 weeks till normal root and shoot system developed.

The rooted plantlets obtained through *in vitro* embryo rescue/ seedlings obtained by *in vivo* seed set were hardened at 3- 4 leaf growth stage by transferring them to 8.5 cm pots containing commercial grade agropeat™ (cocopeat: soilrite: perlite in the ratio of 2:1:1) and kept in Conviron PGV-36 growth chamber maintained at 22 ± 2 °C with 16 h photoperiod and a photon flux of 290 μmol m⁻² s⁻¹. The plantlets were covered with glass jars for initial 3- 4 days and watered with half strength MS medium without sucrose. The jars were removed gradually and the hardened plantlets were subsequently transplanted to the field after 10- 15 days at 4- 5 leaf growth stage. The plantlets obtained through *in vitro* embryo rescue and *in vitro* raised seeds were transplanted in field at the same growth stage and grown as SPP rows.

### 3.2.4 Progeny advancement

All hybrids from the crosses *B. juncea x B. rapa* and *B. juncea x B. carinata* were selfed/ allowed to set open pollinated seeds and backcrossed to their respective female parents to obtain F₂ and BC₁ seeds. Plants raised from F₂ and BC₁ seeds were sown as SPP rows along with their respective female and male parent genotypes as well as infector rows. The plants were selected based on disease response to white rust/ alternaria blight; they were further selfed and backcrossed to their respective female parent to obtain F₃ and F₂BC₁ as well as BC₂F₂ and BC₂ seeds, respectively. The seeds were harvested separately from individual selected plants and were forwarded as individual SPP rows in the next cropping season. The SPPs of BC₁F₂ and BC₂ were sown in the same year at the same time in adjacent blocks opposite to each other. The screening and selection procedure was repeated for BC₁F₂ and BC₂ progenies to select plants with the lowest disease score. Only plants with the lowest disease score were selected for progeny advancement in each generation.

### 3.3 Characterization

The putative hybrids obtained through both *in vitro* embryo rescue and *in vitro* seed set were characterized through molecular markers and were further evaluated for morphological traits under field conditions.
3.3.1 DNA based markers

For molecular characterization, DNA from young leaves of the plantlets was extracted, assessed and then amplified using PCR based ISSR primers. The detailed methodology is given below:

3.3.1a DNA Extraction

Modified CTAB method was followed for DNA extraction (Weising et al., 1995). Lyophilized young leaves (100 mg) were ground to fine powder using mortar and pestle. Silica gel was used as an abrasive material for crushing. Prewarmed (65 °C) 1ml CTAB extraction buffer (Appendix I) was added to the pulverized leaf material and mixed thoroughly. The slurry was transferred to a 2 ml eppendorf tube and incubated at 65 °C for 30 min with intermittent shaking every 10 min. An equal volume (1.0 ml) of chloroform: isoamyl alcohol (24:1; v/v) was added to the DNA samples, mixed thoroughly and centrifuged for 15 min (5000 rpm, 25 °C). The upper aqueous phase was transferred to a fresh eppendorf tube and 30 µl RNase A (10 mg/ml), free of DNase, was added to make the final concentration of 100 µg/ml. The samples were incubated at 37 °C for 30 min and re-extracted with fresh chloroform: isoamyl alcohol. The final aqueous phase was transferred to fresh eppendorf tube with the help of a large bore pipette. Ice-cold isopropanol (0.6 volumes) was added to precipitate the DNA and centrifuged (5000 rpm, 4 °C) for few seconds. The pellet was washed by adding 500 µl washing solution (10 mM ammonium acetate in 70% ethanol) to remove residual CTAB. In order to precipitate the DNA, two volumes of absolute ethanol was added to the samples and the contents were mixed by inversion and stored for 1 h at -20 °C. The pellet was subsequently collected by centrifugation (10 min, 5000 rpm, 4 °C) and allowed to dry. The dried pellet was dissolved in an appropriate volume (200-300 µl) of milliQ water or TE buffer and stored at -20 °C till further use.

3.3.1b DNA Quantification

Qualitative and quantitative assessment of total genomic DNA was performed by subjecting the DNA samples to agarose gel electrophoresis (Sambrook et al. 1989). DNA samples were mixed with loading dye bromophenol blue and loaded in 0.8% agarose gel. Electrophoresis was carried out in 0.5 X TBE buffer at 30 mA for 2-3 h (constant voltage equivalent to 3 V/cm) to allow proper resolution. Subsequently, the gel was stained by EtBr at a final concentration of 0.5 µg/ml and visualized under UV light at short wavelength (254 nm) and photographed under red filter. Serial dilution of uncut Lambda DNA (25-150 ng/µl) was used as standard molecular size marker to quantify genomic DNA.
3.3.1c PCR amplification using ISSR primers

ISSR’s were performed following the modified protocol by Kumar et al. (2001b). A total of eight UBC ISSR primers (synthesized from Microsynth, Switzerland) ranging from 17–20 bases were used for DNA amplification (Table 3.2). Amplification of DNA was carried out in a reaction mixture containing PCR buffer (10 mM of Tris-HCl, 1.5 mM of MgCl₂, 50 mM KCl), 0.25 mM of dNTPs, 0.2 μM primers, 1.0 U Taq DNA polymerase enzyme and 25 ng of DNA in 25μl reaction mixture. PCR amplification was performed in Perkin-Elmer thermocycler programmed as: 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min 30 s, followed by a final extension for 7 min at 72 °C. The amplification product was visualised on 1.5 % agarose gel by staining with EtBr.

Table 3.2 UBC-ISSR primers used for DNA amplification

<table>
<thead>
<tr>
<th>ISSR Primers</th>
<th>Sequence¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC 812</td>
<td>5’GA-GA-GA-GA-GA-GA-GA- A-3’</td>
</tr>
<tr>
<td>UBC 814</td>
<td>′CT-CT-CT-CT-CT-CT-CT-CT- A-3’</td>
</tr>
<tr>
<td>UBC 818</td>
<td>5’CA-CA-CA-CA-CA-CA-CA-G-3’</td>
</tr>
<tr>
<td>UBC 840</td>
<td>5’GA-GA-GA-GA-GA-GA-GA-YT-3’</td>
</tr>
<tr>
<td>UBC 842</td>
<td>5’GA-GA-GA-GA-GA-GA-GA-YG-3’</td>
</tr>
<tr>
<td>UBC 843</td>
<td>5’CT-CT-CT-CT-CT-CT-CT-CT-RA-3’</td>
</tr>
<tr>
<td>UBC 848</td>
<td>5’CA-CA-CA-CA-CA-CA-CA-CA-RG-3’</td>
</tr>
<tr>
<td>UBC S2</td>
<td>5’CTC-TC-TC-TC-GT-GT-GT-GT-3’</td>
</tr>
</tbody>
</table>

¹Y= Pyramidines (C or T) and R = Purines (A or G)

3.3.2 Morphological traits

The F₁ plantlets characterized as hybrids through ISSR markers were further evaluated for their hybridity through morphological traits such as plant height; leaf shape including leaf margins and tip, texture and colour; arrangement of inflorescence; floral morphology including colour of petals; male and female fertility; and seed coat colour. Five plants per individual hybrid were characterized for their morphological traits along with their male and female parent genotypes. Status of female fertility in terms of length of pod and seeds per pod was evaluated using ten randomly collected pods from the main shoot of the individual hybrid plants, while status of male fertility was studied through percent pollen fertility.
3.3.2a Percent pollen fertility
The percent pollen fertility was observed using two stains viz., 2 % acetocarmine (vital stain) and 2 mg/ ml Fluorescein- di- acetate (FDA; a non- vital stain) following the method by Shivanna and Rangaswamy (1992). Pollen from freshly dehisced anthers was tapped on a clean glass slide with a drop of a mixture of acetocarmine/ FDA and glycerine (1:1 v/ v). The mixture with dusted pollen grains was covered with a glass cover slip in a way that the pollen grains were spread equally on the slide under the cover slip. In case where pollen grains were not visible, anthers were squeezed in the drop of stain with the help of a sterilized forceps. Eight slides per genotype were prepared by taking eight randomly collected flowers per genotype and six anthers per flower to prepare one slide. Scoring was done at six arbitrary selected microscopic fields (20 X) per slide, thus 500- 600 pollen grains were scored per genotype for pollen shape and fertility. The pollen grains were scored as fertile when they reflected dark pink or red colour due to acetocarmine observed under light microscope or blue colour due to FDA staining observed under fluorescence microscope using UV with barrier filter (L420) at 405 nm. The colourless pollen grains, that did not absorb any colour with either of the stains, were scored as sterile.

3.4 Evaluation of plants for white rust resistance
The plant progenies derived from B. juncea as female and B. rapa/ B. carinata as male donor were evaluated for their response to white rust at two stages viz., cotyledonary leaf growth stage under controlled conditions and mature leaf growth stage under field conditions. Field isolates of Albugo candida were utilized to screen the progenies for resistance/ high tolerance against white rust.

3.4.1 Fungal isolates
Two A. candida field isolates from B. juncea var. Varuna at Delhi (35 km away from TERI experimental field station; AcBjD) and Pantnagar (250 km away from TERI Experimental station; AcBjP) in Northern India, and one A. candida field isolate collected from B. rapa var. Pusa Gold from Pantnagar (AcBrP) were collected at the initiation of the disease symptoms. The three field isolates were tested in isolation as well as mixtures; one mixture comprising of two B. juncea isolates (AcBjD + AcBjP) and second mixture containing all the three isolates (AcBjD + AcBjP + AcBrP). The different isolates and their mixtures that were utilized to screen the plant progenies at the cotyledonary leaf growth stage under controlled conditions and at the mature leaf growth stage under field conditions are given in Table 3.3.
3.4.2 Screening under controlled conditions

The parent genotypes of *B. juncea* (J0, J7 and M21-1), *B. rapa* (R2 and R3) and *B. carinata* (K) were screened against the above mentioned field isolates of *A. candida* at cotyledonary leaf growth stage. *B. juncea* var. Varuna and *B. rapa* var. Pusa Gold were taken as susceptible check. Seeds of all genotypes were sown in 8.5 cm pots containing autoclaved mixture of soil-less commercial grade agro-peat based compost (Varsha enterprises, Bangalore, India) and kept in plant growth propagators (PGPs; 30.5 x 25 x 10 cm) in Conviron PGV-36 growth chamber at 22 ± 1 °C and 16 h photoperiod, with a photon flux of 290 μmol quanta m⁻² s⁻¹. The experiment was arranged in three replicates, with one PGP in each replicate containing seven pots, one pot of each genotype kept randomly. Fifteen seeds were sown per genotype per replicate that was thinned to 10 seedlings per genotype before inoculation to decrease variability in seedling growth. Water was put at the base of the PGP and seedlings were also sprayed with water using an atomiser to ensure uniform water supply. The 6-7 day old seedlings with fully expanded cotyledonary leaves were taken for inoculation (Plate 3.1). In each replicate, one set containing only parent genotypes and both controls were sprayed with DW without isolate.

### Table 3.3

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Growth stage</th>
<th>Isolates</th>
<th>Source</th>
<th>Host species</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cotyledonary leaf</td>
<td>AcBjP</td>
<td>GBPUA&amp;T, Pantnagar</td>
<td><em>B. juncea</em> var. Varuna</td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td>AcBjD</td>
<td>IARI, New Delhi</td>
<td><em>B. juncea</em> var. Varuna</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>AcBrP</td>
<td>GBPUA&amp;T, Pantnagar</td>
<td><em>B. rapa</em> var. Pusa Gold</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>Mixture 1</td>
<td>AcBjD + AcBjP</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>Mixture 2</td>
<td>AcBjD + AcBjP + AcBrP</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Mature leaf²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td>Mixture 1</td>
<td>AcBjD + AcBjP</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>Mixture 2</td>
<td>AcBjD + AcBjP + AcBrP</td>
<td></td>
</tr>
</tbody>
</table>

*Based on response of isolates at cotyledonary leaf growth stage, only mixture of isolates were used at mature leaf growth stage under field conditions.*

3.4.2a Spore suspension

Preparation of spore suspension and spraying of inoculum on plants was carried out following the method of Singh et al. (1999). Pustules of *B. juncea* and *B. rapa* isolates mentioned in Table 3.3 were tapped from freshly collected infected leaves of their respective genotypes to dislodge zoosporangia in 25 ml autoclaved DW in a sterile glass flask. Extraneous material was removed from the resulting zoosporangia suspension by filtering twice through three layers of muslin cloth.
Density of the suspension was adjusted to $5 \times 10^4$ zoosporangia per ml for each isolate with the help of autoclaved DW using haemocytometer. Inoculum of the isolates was prepared individually in isolation using a laminar flow bench. The mixture of isolates was prepared by mixing an equal volume of the zoosporangia suspension of each individual isolate.

3.4.2b Inoculation

The 6-7 days old seedlings with fully expanded cotyledory leaves were sprayed with DW to remove any agro-peat debris from the surface of cotyledory leaves and allowed to dry for 30 min prior to inoculation. The zoosporangia suspension of individual isolates and their mixtures was then sprayed on to the seedlings till run off using an atomiser. Inoculation was done within 20 min of preparing zoosporangia suspension. After inoculation, all genotypes were placed in individual PGPs, covered with a transparent lid and sealed to ensure 100 % relative humidity for optimum disease infection (Plate 3.1). The PGPs were placed undisturbed under controlled conditions in growth chamber at $18 \pm 1 ^\circ C/15 \pm 1 ^\circ C$ day/ night temperature, in dark for 48 h followed by 16 h photoperiod with a photon flux of $290 \mu \text{mol quanta m}^{-2}\text{s}^{-1}$ for development of pustules.

3.4.2c Disease Assessment

Incubation period for disease initiation, taken as time difference between the time of inoculation and appearance of disease symptoms, was recorded for each genotype against each isolate. The host reaction (interaction phenotype) of the cotyledory leaves was recorded 12 days after inoculation following a 0-7 scale proposed by Leckie et al. (1996; Table 3.4; Plate 3.1).

<table>
<thead>
<tr>
<th>Score</th>
<th>Host Reaction</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No response</td>
<td>Resistant</td>
</tr>
<tr>
<td>1</td>
<td>Light necrotic flecks, no sporulation</td>
<td>Resistant</td>
</tr>
<tr>
<td>2</td>
<td>Heavy necrotic flecks, no sporulation</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>Minute pustules on upper surface of cotyledon</td>
<td>Moderately Resistant</td>
</tr>
<tr>
<td>4</td>
<td>Few pustules on lower surface of cotyledon</td>
<td>Moderately Resistant</td>
</tr>
<tr>
<td>5</td>
<td>Numerous pustules on lower surface of cotyledon</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>6</td>
<td>Large scattered pustules on lower surface of cotyledon</td>
<td>Susceptible</td>
</tr>
<tr>
<td>7</td>
<td>Large coalescing pustules on lower surface of cotyledon</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

*Based on 0-7 scale (Leckie et al., 1996)
3.4.2d Maintenance of fungal culture
The *B. juncea* and *B. rapa* isolates collected from field were maintained on *B. juncea* and *B. rapa* host genotypes (Table 3.3) by inoculating at cotyledonary leaf growth stage under controlled conditions as mentioned in section 3.4.2 a & b. The fresh inoculum of the individual isolates raised on the cotyledonary leaves of their respective host genotype were collected in two forms; one in the form of infected leaf portions in glass vials and other as sporangial dust in gelatine capsules. Both glass vials and gelatine capsules with the inoculum were stored at – 20 °C until further use. The stored inoculum was revived on their host genotype prior to their use for inoculation.

3.4.3 Screening under field conditions
Parent genotypes, hybrids and their advanced progenies were raised in the field without any pre-treatment or any preventive fungicide spray during the cropping season. Based on the response of different isolates screened individually or as a mixture at cotyledonary leaf growth stage under controlled conditions, the parent genotypes, hybrids and their progenies (*F*₂, *F*₃, *F*₂BC₁, BC₁, BC₂, BC₁F₂), raised as SPP rows in the field were screened during four consecutive crop seasons (2001 to 2005) against mixture 1 and mixture 2, following the method of Singh et al. (1999).

3.4.3a Spore suspension
Preparation of spore suspension and spraying of inoculum on the plants was carried out following the method of Singh et al. (1999). Heavily infected portions of the leaves of the isolates, AcBjD, AcBjP and AcBrP, were cut using a sterile scalpel and were shaken thoroughly in autoclaved DW in a beaker to prepare the spore suspension of each isolate separately. Extraneous material was removed from the resulting zoosporangia suspension by filtering twice through 2-3 layers of muslin cloth. The density of the suspension was adjusted to 5 x 10⁴ zoosporangia per ml for each isolate with the help of autoclaved DW using haemocytometer. The spore suspension of isolates AcBjD and AcBjP was mixed in equal proportion to form mixture 1 and the spore suspension of isolate AcBrP was kept, separately.

3.4.3b Inoculation
Spraying of the spore suspension (inoculum) on plants was carried out following the method of Singh et al. (1999). The plant progenies derived from cross *B. juncea x B. carinata* along with their parent lines and infector rows were sprayed with mixture 1, while the plant progenies derived from cross *B. juncea x*
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*B. rapa* along with their parent lines and infector rows were sprayed simultaneously with mixture 1 and isolate AcBrP (equivalent to mixture 2) in the evening when the temperature was low (12 ± 2°C) and humidity was high (> 90%) to ensure optimum disease severity on the plants. The field-transplanted hybrids were sprayed with white rust inoculum after 20 days of transplanting, while the seed raised plant progenies were sprayed with white rust inoculum 50 DAS.

3.4.3c Disease Assessment

The field-transplanted hybrids were evaluated three times; at the mature leaf growth stage and after 40 and 60 days after spraying, to study the progression of disease, while seed raised plant progenies, parent genotypes and the controls were evaluated three times at:

i. 75 DAS - Peak flowering stage (PFS)

ii. 90 DAS - End of flowering stage (EFS)

iii. 110 DAS - Pod bearing stage (PBS)

All plants in the field were tagged and numbered. Three leaves per plant i.e. 3rd, 4th and 5th leaf from base of the plant were screened for all plants in a progeny and the same leaves were screened at the three time intervals. Disease index (DI) was assessed on a modified 0-5 scale based on Conn et al. (1990) as mentioned in Table 5 (Plate 3.2). The plants with a DI of less than 1.0 were selected and forwarded to the next generation.

<table>
<thead>
<tr>
<th>Score</th>
<th>Host Reaction</th>
<th>Plant Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visible disease symptoms</td>
<td>Resistant</td>
</tr>
<tr>
<td>1</td>
<td>1-5% area covered with disease symptoms</td>
<td>Resistant</td>
</tr>
<tr>
<td>2</td>
<td>6-10% area covered with disease symptoms</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>11-25% area covered with disease symptoms</td>
<td>Moderately resistant</td>
</tr>
<tr>
<td>4</td>
<td>26-50% area covered with disease symptoms</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>5</td>
<td>&gt;50% area covered with disease symptoms</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

*Based on 0-5 scale (Conn et al., 1990)

3.4.4 Correlation between cotyledonary and mature leaf growth stage screening

Some of the BC₂ seeds from all cross combinations of cross *B. juncea* x *B. rapa* / *B. carinata* were utilized to study the relationship between the response of plants to white rust under controlled and field conditions. For the correlation
analysis, the seedlings that were screened against a mixture of isolates at cotyledonary leaf growth stage under controlled conditions were transplanted in field and screened again at mature leaf growth stage under epiphytotic conditions.

Out of the total seeds harvested, ten seeds per genotype were raised as mentioned in Section 3.4.2 for screening at cotyledonary leaf growth stage. The experiment was set such that PGP having progenies from *B. juncea* and *B. rapa* contained both *B. juncea* var. Varuna and *B. rapa* var. Pusa Gold as controls, while PGP containing *B. juncea* and *B. carinata* genotypes included *B. juncea* var. Varuna as control. Due to availability of limited seeds, the experiment was arranged in two replicates. The stored inoculum of the isolates, AcBjD, AcBjP and AcBrP, was revived on the cotyledonary leaf growth stage under controlled conditions using the host species (Table 3.3) as mentioned in section 3.4.2 a-b.

Freshly revived inoculum of the isolates was utilized to prepare mixture 1 and mixture 2 to inoculate the seedlings at cotyledonary leaf growth stage derived from cross *B. juncea* x *B. carinata* and *B. juncea* x *B. rapa*, respectively, under controlled conditions as well as mature leaf growth stage under field conditions as mentioned in section 3.4.2 and3.4.3. Individual seedlings were screened after 12 days of inoculation on a 0-7 scale (Table 3.4; Leckie et al., 1996). Approximately 30-35 DAS, the screened seedlings tagged to keep a track at mature leaf growth stage were transplanted in field. They were inoculated again at 50 DAS under field condition with their respective mixture of isolates as mentioned in section 3.5.3 and evaluated after 75, 90 and 110 DAS on a 0-5 scale (Table 3.5; Conn et al., 1990). The response of plants to white rust was compared and analysed at the two-growth stages viz., cotyledonary leaf growth stage under controlled conditions and mature leaf growth stage under field conditions.

### 3.5 Evaluation of plants for alternaria blight

The plant progenies obtained from the cross *B. juncea* x *B. carinata* were evaluated for their response to alternaria blight by screening at mature leaf growth stage under controlled and field conditions. Field isolates of *Alternaria* were utilized to screen the progenies for resistance/ high tolerance against alternaria blight.

#### 3.5.1 Fungal isolates

Leaves of *B. juncea* var. Varuna infected with alternaria field isolate (s) at IARI, New Delhi (35 km away from TERI Experimental field station) in Northern
India were collected at the initiation of the disease symptoms. The infected leaf portions were utilized to prepare conidial suspension or were used to produce fungal toxin. The conidial suspension and fungal toxin were utilized to screen the plant progenies at mature leaf growth stage under field and controlled growth conditions, respectively. The preparation of these two is given below:

3.5.1a Conidial suspension

The infected *B. juncea* var. Varuna leaves were collected on the day of inoculation only. Infected portions of the leaves were shaken thoroughly with the help of sterilized rod and filtered through three layers of sterilized muslin cloth to remove extraneous material. Density of conidia in the suspension was adjusted to $1.5 \times 10^4$ conidia per ml with the help of haemocytometer before inoculation. The conidial suspension prepared for screening under controlled conditions was centrifuged at 1800 rpm for 5 min before adjusting the density of conidial suspension. Conidial suspension was freshly prepared an hour before inoculation.

3.5.1b Fungal toxin

The method proposed by Bains and Tewari (1987) was followed for toxin production with minor modifications. To initiate fungal culture, infected leaf areas of *B. juncea* var. Varuna surrounded with healthy leaf portions were cut and surface sterilized with 0.01 % HgCl$_2$ for a minute and rinsed thoroughly with distilled water for 2-3 times. Sterilized leaf portions were blotted on a sterile filter paper and put on V8 juice agar medium supplemented with 50 µg/ L Rose Bengal (Appendix III) in a 90 mm disposable petri plate or culture glass tube. The petri plate/ culture glass tube was sealed and kept undisturbed at $22 \pm 2$ ºC with 12 h photoperiod for 7 to 10 days, till the pathogen sporulated profusely (Plate 3.1).

The sporulating fungal culture was mixed thoroughly in sterilized DW, filtered through three layers of sterile muslin cloth and centrifuged at 1800 rpm for 5 min. The density of the conidial suspension was adjusted to $5 \times 10^4$ conidia/ ml. One ml of this suspension was added to 100 ml of V8 juice liquid medium in 250 ml Erlenmeyer conical flask and kept in dark on a gyratory shaker at 120 rpm at $22 \pm 2$ ºC for 14 days. V8 juice liquid medium without the conidial suspension was used as control. After 14 days, the fungal broth was filtered in Roux bottles through two layers of Mira cloth (Calbiochem corporation, USA) followed by two layers of Whatman filter paper No. 1. Six g/ L charcoal was added to the filtered suspension and placed on a rotator at 110 rpm for two days at $4$ ºC (Plate 3.1). The charcoal suspended suspension was then centrifuged at 1800 rpm for 10
min and supernatant was discarded. The pellet, after rinsing once with distilled water was extracted thrice with analytical grade ethyl acetate and the fractions were pooled. The pooled fractions were flash evaporated to 20 % of original volume at 45 °C and stored at 4 °C until use.

3.5.2 Screening under controlled conditions

The parent genotypes were screened under controlled laboratory conditions using detached leaf method (Vishwanath and Kolte, 1997 b). Both conidial suspension and fungal toxin were utilized to screen the leaves for response of plants to fungal toxin and conidial suspension. For this, seeds of parent lines and B. juncea susceptible check var. Varuna were sown in 15 cm plastic pots (one plant per pot) with autoclaved commercial grade agropeat™. Ten plants per genotype were raised in PGV growth chamber at 22 ± 2 ºC with 16 h photoperiod.

3.5.2a Inoculation

Third/ fourth fully expanded leaf from base of 30-day-old plants were detached with the help of sterilized blade, washed gently with DW and air dried on a sterile blotting paper under a laminar air flow. Three leaves per plant were inoculated with fungal toxin and conidial suspension. Each leaf was inoculated at four points, two on either side of the mid rib. The two points on right side of the mid rib were inoculated with the conidial suspension and the other two points on left side of the mid rib were inoculated with fungal toxin. The fungal toxin stored at 4°C was diluted 10³ times prior to inoculation. Precisely taken 10 µl of diluted toxin and 10 µl drop of freshly prepared conidial suspension, with a density of 1.5 x 10⁴ conidia/ ml, were applied at the four points of the leaf, as mentioned above. One leaf in each replicate was inoculated with 10 µl of liquid V8 juice medium broth as control. The inoculated leaves for each treatment were kept separately inside a petri plate lined with moist filter paper and kept at 22 ± 2 °C with a 12 h photoperiod for 6-7 days. The experiment was repeated thrice. The observation for development of disease symptoms on the detached leaf was taken after 7 days of inoculation.

3.5.2b Disease Assessment

The response of detached leaves to fungal toxin was assessed after a week on a 0-5 scale following the method of Vishwanath and Kolte (1997 b) as presented in Table 3.6.
Table 3.6 Scale of disease assessment used for alternaria blight resistance under controlled conditions

<table>
<thead>
<tr>
<th>Score</th>
<th>Host Reaction</th>
<th>Plant Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visible chlorosis</td>
<td>Resistant</td>
</tr>
<tr>
<td>1</td>
<td>Slightly visible small chlorotic area</td>
<td>Resistant</td>
</tr>
<tr>
<td>2</td>
<td>Distinctly visible small chlorotic area and/or few small necrotic lesions</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>Large chlorotic areas and/or large necrotic lesions</td>
<td>Moderately resistant</td>
</tr>
<tr>
<td>4</td>
<td>Severe chlorotic areas with many coalescing large necrotic lesions</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>5</td>
<td>Large expanding chlorotic areas often associated with scorching of the leaves</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

*Based on 0-5 scale (Vishwanath and Kolte, 1997 b)*

The response of plants to the conidial suspension was assessed following the modified 0-5 scale based on Conn et al. (1990; Table 3.5). The phenotypic interaction for both fungal toxin and conidial suspension was scored as resistant (0-2), moderately susceptible (3-4) and susceptible (>4).

### 3.5.3 Screening under field conditions

Based on the response of parent genotypes to conidial suspension and fungal toxin at mature leaf growth stage under controlled conditions, the parent genotypes, hybrids and their progenies (F₂, F₂BC₁, BC₁, BC₂, BC₁F₂), raised as SPP rows in the field without any pre-treatment or preventive fungicide spray, were screened 40 DAS using conidial suspension during four consecutive crop seasons. The conidial suspension was prepared as mentioned in section 3.5.1 without centrifugation and sprayed on plants in the evening within one hour of its preparation. The plants were screened for the initiation of disease after 3-4 days of spraying of conidial suspension (inoculum).

All plants in a progeny were evaluated by screening mature 3rd, 4th and 5th leaf from base of each individual plant at three stages of plant growth; 75, 90, 110 DAS on a 0-5 scale (Table 3.5; Plate 3.2). The plants with a DI of less than or equal to 1.0/2.0 were selected and forwarded to next generation in each cropping season. Besides screening the plants at mature leaf growth stage, they were also evaluated at physiological maturity, by screening ten randomly selected pods from the main shoot of each plant following the 0-5 scale as mentioned in Table 3.5.

### 3.6 Evaluation for agro-morphological and Biochemical traits
The parent genotypes and the derived progenies (F₂, BC₁, F₂BC, BC₂ and BC₁F₂) from crosses J0R2, J0R3, J7R2, J7R3, J0K, J7K, M21-1K and crosses J0K, J7K, M21-1K, selected for resistance/ high tolerance to white rust and alternaria blight, respectively, were evaluated for their agro-morphological and biochemical traits.

3.6.1 Agro- morphological traits

All plant progenies were characterized for agro- morphological traits such as plant height; branching pattern; leaf shape including leaf margins, tip, texture and colour; arrangement of inflorescence; floral characteristics including colour of petals, anther structure and seed coat colour. Besides this, the plants from BC₁F₂ and BC₂ progenies in all crosses selected for desirable disease resistant traits (DI ≤ 1.0) were also evaluated for important yield attributing agronomic traits, namely, days to 50% flowering, days to maturity, distance from base to first branch, number of primary branches, number of secondary branches, pods on main shoot, length of pod, seeds per pod and 1000 seed weight. Five plants selected from each progeny were evaluated for agro- morphological traits. Length of pod and number of seeds per pod was calculated by taking mean of ten randomly collected pods from the main shoot.

3.6.2 Biochemical evaluation

The plants selected, for resistance/ high tolerance to white rust and alternaria blight from F₂, BC₁, F₂BC, BC₂ and BC₁F₂ progenies of crosses J0R2, J0R3, J7R2, J7R3, J0K, J7K, M21-1K, were analysed for the biochemical parameters; fatty acid profile, total glucosinolate content and oil content.

3.6.2a Fatty acid analysis

The fatty acid profile was analysed following the method of Kaushik and Agnihotri (1997) using Fatty Acid Methyl esters (FAMEs) analysis through Gas Liquid Chromatograph (GLC). Selfed seeds of each genotype were utilized in two replicates to analyse the FAMEs. About fifteen seeds were crushed using mortar and pestle and taken in a test tube with screw cap. 1 ml of methylating reagent [methanol, acetyl chloride, and benzene; 20: 1: 4 (v/v)] was added to each test tube for extraction and methylation of the oil. The tubes were capped tightly and heated in a water bath at 70 °C for 1 h. After cooling the tubes at room temperature for about 30 min, 2 ml of n- hexane was added in each tube and centrifuged at 5000 rpm for 10 min. The supernatant was collected in a fresh tube and the solvent present in the supernatant was allowed to evaporate for overnight at room temperature leaving behind methyl esters. The methyl esters
were stored at 4 °C until analysed. The preparation of the sample for analysis of fatty acid composition through GLC is depicted in Figure 3.1.

For analysis of fatty acid composition, the stored methyl esters were dissolved in 250 µl hexane and 1 µl of sample was injected into the gas chromatograph fitted with 1.8 m × 2 mm (length × outer diameter) stainless steel packed column [mixture of SP 2300 (2%) and SP 2310 (3%) on Chromosorb W] having mesh size of 100-120. The injector and detector temperatures were maintained at 240 °C while oven temperature was kept at 210 °C. The flow of carrier gas nitrogen was maintained at 30 ml/ min. The relative proportion of fatty acids were analysed on the basis of retention time and peak area.

![Figure 3.1 Extraction of fatty acid methyl esters](image)

3.6.2 b Glucosinolate Analysis

The glucosinolate analysis was performed following the method of Kumar et al. (2004) using Sodium tetra chloro-palladate (Pd)- glucosinolate complex method. Open pollinated seeds were utilized in two replicates to analyse glucosinolate content. Approximately, 500 mg whole seed without any inert material was crushed using mortar and pestle and 200 mg crushed seed was taken in a tube with screw cap. 300 µl of 80 % methanol was added to each sample with the help of micropipette. The tubes were then capped tightly and
heated in a water bath at 80 °C for 5 min to deactivate myrosinase activity in seeds. 2 ml of DW was added into each sample and the mixture was heated again in a water bath for 15 min at 80 °C. The tubes were cooled at room temperature for an hour and centrifuged at 5000 rpm for 5 min to extract glucosinolate in water. The supernatant was transferred in a fresh tube with the help of eppendorf pipette and used for analysis. Sample without crushed seed was used as a blank. The sample preparation for analysis of total glucosinolate content is depicted in Figure 3.2.

The sample thus prepared was used for analysis of total glucosinolate content using ELISA Reader. A sample of 5 μl was taken into each well in a microtitre plate and two wells per sample per replicate were analysed. 300 μl of 2 mM sodium tetra-chloro palladate solution was added into each sample. The microtitre plate was then covered with a lid and incubated in oven at 70 °C for 30 min. The optical density was recorded in ELISA Reader at 405 nm.

**Figure 3.2** Estimation of total glucosinolate content
30 min. Optical density (OD) of samples in the microtitre plate were recorded immediately after 30 min using ELISA Reader at 405 nm (A405). The absorbance (OD values) of the sample was converted to μmol/ g using two equations based on the values of OD (Kumar et al., 2004). Equation 1 or 2 was used for the samples having an OD of less than or equal to 0.8 or more than 0.8, respectively.

Equation 1 = [A405 – 0.046] × 82 and Equation 2 = [A405 – 0.169] × 115.74

3.6.2c Oil content

The open pollinated seeds, of the plants selected for resistance/ high tolerance to white rust and alternaria blight, were analysed for seed oil content using a pre-standardized (AOCS Procedure Am 1-92, 1999) Near Infrared Spectroscopy (NIRS) monochromator in reflectance mode. The calibrations for percent oil content were developed for different Brassica species (Prem et al., unpublished data) using the appropriate reference method, namely, Nuclear Magnetic Resonance (Tiwari et al., 1974). Approximately, a 500 mg of whole seed sample, free from any inert material, of the selected genotypes was analysed using a standard sample cup and the percent oil content was recorded. All samples were analysed in two replicates.

3.6.2d Correlation between disease index and biochemical profile

The plants from BC1 progeny from all cross combinations were used to study the correlation between DI and biochemical parameters. The plants were segregated into two categories on the basis of their DI (0-5 scale; Table 3.5): resistant/highly tolerant (DI = 0.0–2.0) and susceptible (DI = 3.0–5.0), and were analysed in two replicates. The selfed and the open pollinated seeds harvested from these plants were analysed for the biochemical traits mentioned in section 3.6.2 and a comparative analysis was carried out to study the relationship between disease index and biochemical parameters.

A brief outline of the methodology/strategy followed to identify B. juncea type plants with resistance to white rust/alternaria blight along with desirable agromorphological/biochemical parameters is given in Figure 3.3.

3.7 Statistical Analysis

3.7.1 Hybridization and characterization

A two-tailed t-test was applied to study the efficiency of in vitro techniques and in vivo seed set using software package Co Stat (CoHort, Berkeley, California). For interpretation of DNA fingerprinting results, a binary matrix was made by scoring the data for the presence (1) and absence (0) of bands across genotypes.
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Female parent 
(B. juncea) × male donor 
(B. rapa/ B. carinata)

In vitro

• Ovary culture
• Ovule culture
• Sequential embryo

Putative F₁’s raised and 50% seeds 50% sown in field

DNA

Hybrids rooted, hardened and

Morphological characterization (% pollen/ female

Screened for white rust and alternaria blight

Resistant/ highly tolerant plants selfed/

F₂ and BC₁ plant progenies

Screened three leaves/ plant at 75, 90, 110 DAS and Step for white rust and alternaria blight under epiphytotic conditions;

B. juncea type resistant/ highly tolerant plants selfed/ backcrossed;

Some of BC₁F₂ and BC₂ seeds used for

• Correlation between DI and biochemical traits
• Correlation b/w response to white

Remaining F₂BC₁, BC₁F₂ and BC₂ seeds raised in field

B. juncea type plants, DI < 1.0 for white rust, DI < 2.0 for alternaria blight selected and advanced

Selected plant progenies evaluated/ characterized for agronomic and biochemical traits

Figure 3.3 Strategy followed for resistance
On the basis of a binary matrix, a similarity matrix was constructed using Jaccard’s coefficient (Jaccard, 1908). The similarity matrix was subjected to Sequential Agglomerative Hierarchal Nested Clustering (SAHN) and the phenotypic dendrogram was constructed by the method of Sneath and Sokal (1973) employing Unweighted Pair Group Method of Arithmetic Averages (UPGMA) to group individuals into discrete clusters. Molecular data was analysed using statistical software package NTSYS version 2.0 (Rohlf et al., 1971).

3.7.2 Disease evaluation

The average DI of plants for white rust and alternaria blight was recorded by screening three leaves (3rd, 4th and 5th leaf from base) of each plant. Depending on the number of plants available, 5-20 plants per progeny were evaluated to calculate the mean DI of the SPP. Single Factor Analysis of Variance (ANOVA) was carried out to study the difference between the SPP rows of an individual hybrid of a cross, difference among the individual hybrids of a cross and difference between the different crosses. The significant differences, if any, calculated using F-test among the progenies and the crosses means was analysed using Least Significant Difference Test (LSD) at $p = 0.05$. Since the number of plants in each progeny was not constant, the LSD values for difference between the two progenies were calculated separately following the formula given by Gomez and Gomez (1984):

$$t_{a (0.05)} \left[ \text{Error mean square (1/n}_i^{th} + 1/m_j^{th} \right]^{0.5}$$

Where, $n$ and $m$ is the number of plants in $i^{th}$ and $j^{th}$ progeny/cross, respectively. The BC$_1$ plant progeny germinated under field conditions was also assessed to study the segregation of resistant and susceptible plants using Chi square analysis ($\chi^2$). Correlation coefficient analysis was carried out to study relationship between the progressions of disease with the increasing DAS in all generations using software package Co Stat.

3.7.3 Agro- morphological and Biochemical evaluation

The variation between various agro- morphological traits and biochemical profile of different progenies was evaluated through Analysis of Variance using Least Significant Difference (LSD) through Co Stat (CoHort, Berkeley, California). The composition of different fatty acids and glucosinolate content of the selected progeny was compared with their female and male parents using paired t-test assuming unequal variances. The relationship between disease response of the plants and their biochemical parameters was assessed through
Materials & Methods

correlation coefficient analysis. The disease index of the plants (0-5 scale) taken as dependent variable and the biochemical parameters as independent variable were analysed as such for correlation analysis and were also transformed using Log transformation and subjected to correlation coefficient analysis in order to look for any meaningful relationship using software package Co Stat.