# 3. MATERIALS AND METHODS

### 3.1. Plant materials

In the present study, pointed gourd plants were used as experimental host for studies on plant-pathogen interactions. The details of the obtained plant materials have been described in the following paragraphs.

#### 3.1.1. Collection of pointed gourd plants

Healthy pointed gourd roots (of 'Swarna aloukik' variety) were collected from the local agricultural fields of Siliguri (District Darjeeling), West Bengal, India. As 'Swarna aloukik' variety grow well in the agro-climatic conditions of sub-Himalayan West Bengal and are widely cultivated by the farmers in the present study area, the variety was chosen (Fig. 3.1). Healthy pointed gourd fruits were also collected from the farmers for experimental purpose whenever required.

#### 3.1.2. Maintenance of pointed gourd plants

i. Pointed gourd plants were cultivated both in pots and fields of the experimental garden of the Department of Botany, University of North Bengal.

ii. Round earthen pots (30 cm diameter) were filled with 3.0 kg of soil mixture. The soil mixture was prepared by adding 0.5 kg of sun dried cow dung manure to 2.5 kg of fine dry soil.

iii. The same mixture was also used for preparing the experimental plots in the field for the cultivation of the pointed gourd plants.

iv. Roots were surface sterilized with 0.1 % sodium hypochlorite solution and then washed thrice with sterile distilled water.

v. Sterilized roots were then sown in soil at one inch below the soil surface in the pots as well as in pots of experimental field. In field condition plants were grown at a distance of one meter between row to row and also between plant to plant.

vi. Watering was done on every alternate day to maintain the moist condition of the soil. Weeds were removed regularly at an interval of 10 days.

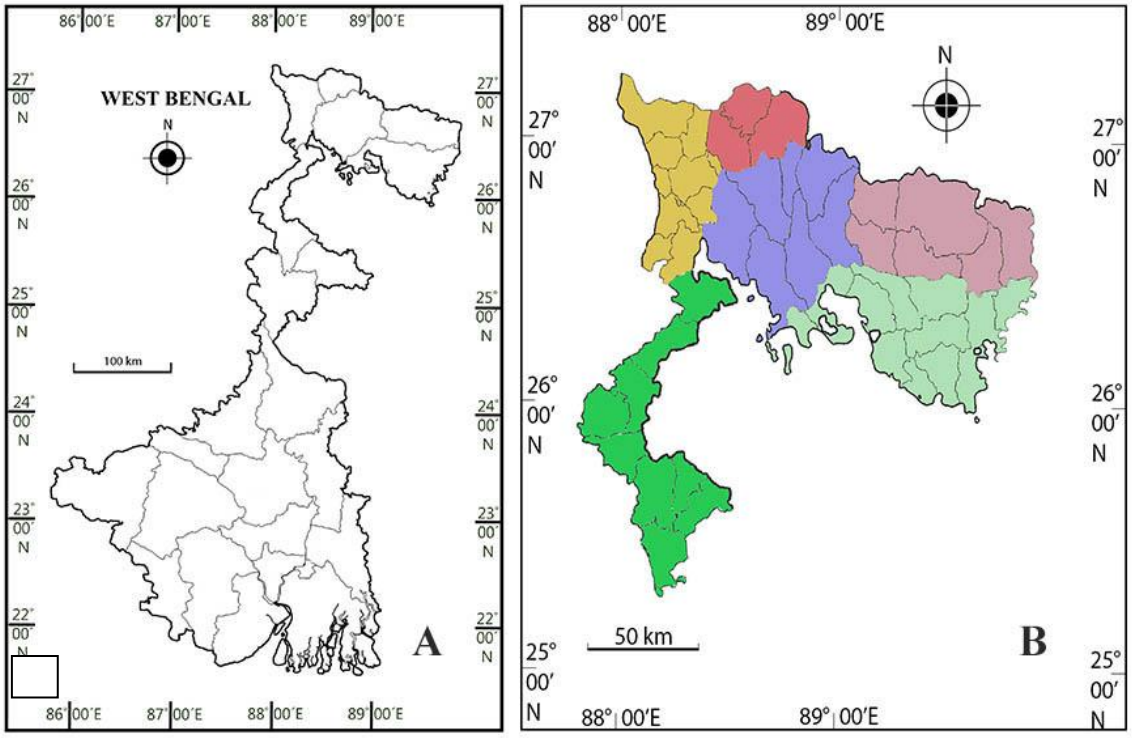


**Fig. 3.1:** (A) Map of West Bengal. (B) Districts of northern part of West Bengal (The present study area) where pointed gourd is grown as an important vegetable crop.

## 3.2. Pathogenic fungi

### 3.2.1. Field survey and collection of disease samples

A field survey was conducted on the occurrence of diseases in pointed gourd plants in six districts (Darjeeling, Jalpaiguri, Kalimpong, Alipurduar, Coochbehar and Uttar Dinajpur) of sub-Himalayan West Bengal (Fig. 3.1). Altogether 20 surveys were done during 2015–2018 where leaves, tender stems and fruits were observed randomly. All such survey was done in large open cultivation fields. Numbers of affected leaves and fruits were counted and the percentage of infection was computed.

Pointed gourd leaves showed different types of disease symptoms like leaf whitening, brown spot of leaves, leaf necrosis and leaf blight. Fruits also showed typical anthracnose symptoms and fruit rot of various intensities. Both the diseased leaves and fruits were randomly collected from different pointed gourd cultivation fields located in Kharibari, Falakata, Tufanganj, Islampur, Phansidewa, Dhundiajor, Kranti, Dhupguri, Dinhata, Salkavita, Tambari, Gorongdanga and Guabari of sub-Himalayan West Bengal (Fig 3.2

& 3.3). Several samples were also collected in separate sterilized zip-packs and labeled accordingly (Table 3.1). The zip-packs with samples were then brought to the Molecular Plant Pathology Laboratory of University of North Bengal and stored at 4°C for further studies.

### 3.2.2. Isolation of fungi

i. Pointed gourd leaves and fruits showing disease symptoms were taken and the affected parts were carefully cut out with a sharp blade.
ii. The dissected diseased portions were then surface sterilized with 0.1 % sodium hypochlorite (NaOCl) solution for 1 minute and subsequently washed with sterilized distilled water for four consecutive times to remove traces of NaOCl.
iii. The surface sterilized diseased portions were then cut into 5 mm small pieces with heat sterilized blade under aseptic conditions.
iv. The pieces were then transferred into sterilized potato dextrose agar (PDA) slants in three replications.
v. Some fresh healthy plant parts were also cut into small pieces and after surface sterilization, transferred into PDA tubes as control.
vi. The tubes were then kept in an incubator at 28°C for 5 days (Thiyam and Sharma, 2013).

### 3.2.3. Maintenance of stock cultures

Cultures of isolated fungi were maintained in freshly prepared sterile PDA slants. Cultures were stored either at 4°C or at room temperature. Subcultures were prepared from stored stock cultures and used for all experimental purposes as and when required.

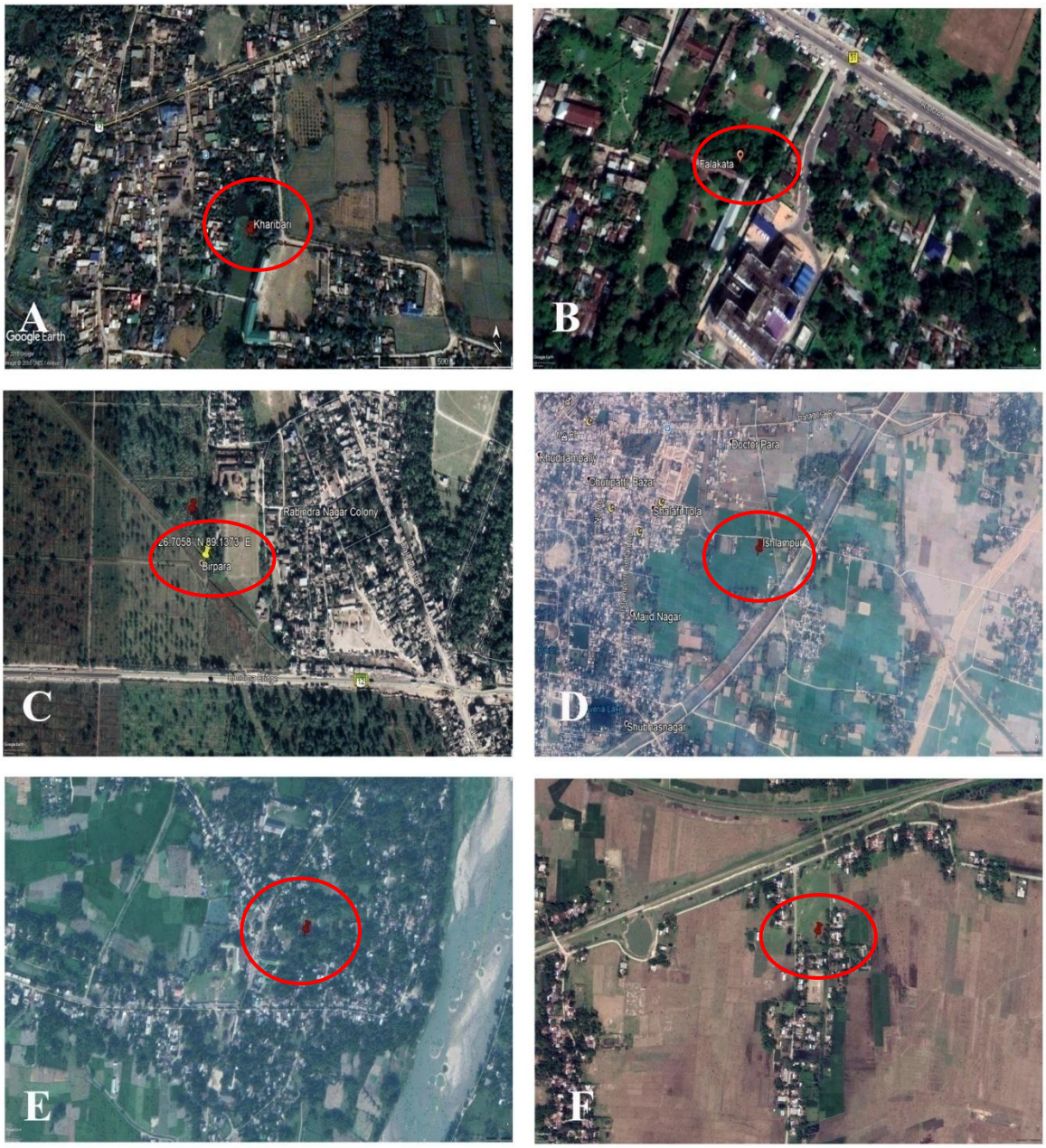


**Fig. 3.2:** Map of regions of collection of infected plant samples from different districts of North Bengal: (A) Kharibari, Siliguri subdivision of Darjeeling District. (B) Falakata, Alipurduar District. (C) Birpara, Alipurduar District. (D) Islampur, Uttar Dinajpur District. (E) Phansidewa, Siliguri subdivision of Darjeeling district and (F) Guabari, Siliguri subdivision of Darjeeling district. The red pins showing the collection spots.

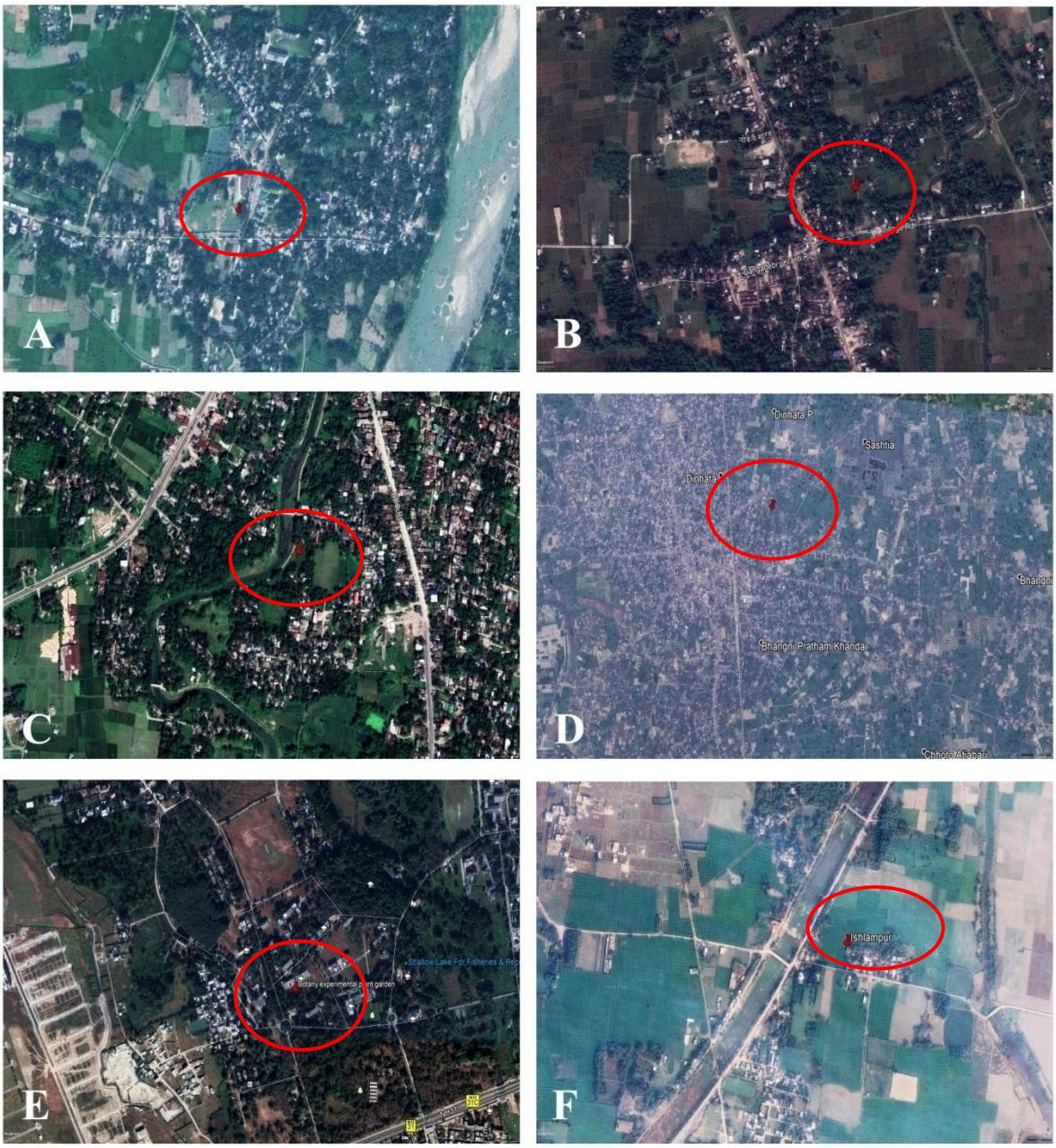


**Fig. 3.3:** Map of regions of collection of infected plant samples from different districts of North Bengal: (A) Phansidewa, Siliguri subdivision of Darjeeling District. (B) Kranti, Jalpaiguri District. (C) Dhupguri, Jalpaiguri District. (D) Dinhata, Cooch-behar District. (E) Salkavita, Darjeeling District and (F) Islampur, Uttar Dinajpur District. The red pins showing the collection spots.

**Table 3.1. Region of collection of fruit and leaf samples of pointed gourd.**

| Place of sampling with District in parentheses | Disease | Code assigned to the fungal isolate | Time of sampling | GIS locations | |
|---|---|---|---|---|---|
| | | | | Latitude | Longitude |
| Kharibari (Darjeeling) | Leaf spot | KHBR | July-2015 | 26.5540° N | 88.1916° E |
| Falakata (Alipurduar) | Leaf spot | PGAL | April-2016 | 26.5175° N | 89.2039° E |
| Birpara (Alipurduar) | Leaf blight | PGALD | April-2016 | 26.7058° N | 89.1373° E |
| Islampur (Uttar dinajpur) | Necrosis | PGISH | March-2017 | 26.2554° N | 88.2009° E |
| Phansidewa (North) (Darjeeling) | Fruit rot | PG-Ph | May-2017 | 26.5909° N | 88.3704° E |
| Guabari (Darjeeling) | Fruit rot | PG-GUA | June-2017 | 26.6016° N | 88.3745° E |
| Phansidewa (South) (Darjeeling) | Antracnose | PG-Pha | Mar-2018 | 26.5859° N | 88.3697° E |
| Kranti (Jalpaiguri) | Leaf spot | PG-Kra | May-2018 | 26.7144° N | 88.7157° E |
| Dhupguri (Jalpaiguri) | Leaf spot | PG-WD1 | May-2018 | 26.5821° N | 89.0051° E |
| Dinhata (Coochbehar) | Leaf spot | PG-WD2 | May-2018 | 26.1291° N | 89.4695° E |
| Salkavita (Darjeeling) | Leaf spot | PG-Gar | June-2018 | 26.7136° N | 88.3507° E |
| Phansidewa (East) (Darjeeling) | Leaf spot | PG-Tau | July-2018 | 26.5891° N | 88.3743° E |
| Islampur (Uttar dinajpur) | Leaf spot | PG-Ish | July-2018 | 26.2476° N | 88.2079° E |

### 3.3. Pathogenicity test of isolated fungi

#### 3.3.1. Inoculum preparation

i. Each isolate was grown in PDA plates for 10–12 days at 28°C for adequate sporulation.
ii. Sterile distilled water was added aseptically to the culture plates. The conidia were detached from the mycelia by gently brushing the surface of the mycelia mat with inoculation needle.
iii. The suspension was filtered through three layers of muslin for removing fragments of mycelia.
iv. The concentration of the conidial suspension was measured using haemocytometer and the final concentration was adjusted to 1 x $10^6$ spores $ml^{-1}$.
v. Conidial suspensions prepared from the fungal isolates were used as inoculums.

#### 3.3.2. Disease induction

##### 3.3.2.1. Detached leaf inoculation technique

Artificial inoculation of detached leaves with test pathogen was performed following the detached leaf inoculation technique of Dickens and Cook (1989) with certain modifications.

i. To perform the experiment fresh young fully expanded and detached pointed gourd leaves were placed on trays lined with moist blotting papers.
ii. The leaves in the trays were inoculated with spore suspension of the pathogen.
iii. Initially two to four wounds (light scratch of 2 mm length) were made on the adaxial surface of each leaf with the help of a sterile, sharp needle.
iv. Twenty microliters drop of spore suspension (bearing about 1x$10^6$ conidia/ml) of test pathogen prepared from 10–12 days old cultures, and were placed on the wounds of each leaf with a micropipette.
v. In control sets, drops of sterile distilled water were placed on the leaves.

vi. Each tray was covered with a glass lid and sealed with petroleum jelly in order to maintain the required moistures inside the trays during incubation. The whole experiment was repeated thrice.

**3.3.2.2. Whole plant inoculation technique**

Disease induction in whole plants was also done following the method of Dickens and Cook (1989). For this,

i. Well established pointed gourd plantlets (1-month-old) grown in pots were taken.
ii. The experimental plants were inoculated by spraying a pure conidial suspension (containing 0.05% tween-20) prepared from each fungal isolate.
iii. Each fungus was inoculated separately in separate experimental sets. Three plants were taken in each set.
iv. Plants in the control set were sprayed by sterile distilled water.
v. Immediately after inoculation, the pots were shifted to perforated transparent polythene chambers, previously mist-sprayed with sterile distilled water for maintaining high humidity.
vi. After two days, the plants were removed from the chamber and grown in green house under natural light and temperature with normal soil surface watering. Humidity was maintained by spraying sterile distilled water at intervals. The whole experiment was repeated thrice.

**3.3.2.3. Disease induction in fruits**

i. Healthy fruits (60–70g approx.) were freshly harvested from the cultivation fields and were thoroughly washed with sterile distilled water.
ii. These were pat dried with sterile blotting paper and placed carefully on moist blotting paper laid on tray.
iii. For inoculation, wounds in the form of two light scratches (1 cm) were made on the surface of the fruit using a sterile scalpel.
iv. Conidial suspensions prepared from each fungal isolate were placed carefully on the scratches.

v. Sterile cotton plugs lightly moistened with conidial suspension were used to cover the inoculated wounds. Eight fruits were inoculated by each isolate.

vi. A set of four fruits that received sterile distilled water instead of conidial suspension served as control.

vii. Each tray was covered with a glass lid and sealed with petroleum jelly in order to maintain the required moisture inside the trays during incubation.

viii. The inoculated fruits were observed for appearance of disease symptoms daily. Disease assessment was done every alternate day and the data were recorded. The whole experiment was repeated thrice.

### 3.3.3. Disease assessment

#### 3.3.3.1 Assessment of disease in detached leaves inoculated by pathogen:

Disease assessment was done after 24, 48 and 72h post-inoculation. Percentage of lesions was calculated as follows:

$$\frac{\text{Total no. of lesions formed}}{\text{Total no. of inoculation drops}} \text{ X } 100$$

In addition, mean diameter of lesions in millimeter were also recorded after 24, 48 and 72h of inoculation.

#### 3.3.3.2. Assessment of disease in leaves of whole plants

i. Assessment of disease in leaves of pointed gourd seedlings was done after 2, 4, 6, 8 and 10 days of inoculation.

ii. The diameters of each lesion were measured and number of lesions developed on the leaves was noted.

iii. The results were computed following the method of Sinha and Das (1972). The sizes of lesions were categorized into four groups and a value was assigned to each group as follows:

- ❖ Very small-restricted lesions of 1–2 mm diameter = 0.1
- ❖ Lesions with sharply defined margins of 2–4 mm diameter = 0.25
- ❖ Slow spreading lesions of 4–6 mm diameter = 0.5

- ❖ Spreading lesions of variable size (beyond 6 mm in diameter) with diffused margin= 1.0

iv. The number of lesions in each group was multiplied by the value assigned to it and the sum total of such values was noted.

v. Disease index was calculated as the mean of observations on three plants per treatment and data was computed as mean disease index per plant.

**3.3.3.3. Assessment of disease in Fruits**

In fruits post-inoculation symptoms were assigned into four different disease grades. Where no yellowing was found, the grade was assigned as '0'; when yellowing just started it was assigned as '+'; when yellowing turned to orange then it was assigned as '++'. Finally, when the fruit became brown, necrotic it was graded as '+++'.

**3.3.4. Verification of Koch's postulations**

Koch's postulations were verified in order to confirm that the fungal isolates were the causal organisms of the diseases in pointed gourd. For this, each fungus was tested for its ability to induce the disease in healthy leaves and fruits. Thereafter, the pathogens were re-isolated from the experimentally diseased tissues and their identities were matched with the fungal isolates.

Healthy leaves were inoculated by conidial suspension ($1 \times 10^6$ conidia $ml^{-1}$) prepared from 10–12 d old cultures of the fungal isolates in PDA plates as stated earlier in section 3.3.2. After 3–7 days of inoculation, when disease symptoms were evident on the leaves, infected portions of the leaves were cut out with a sterile sharp blade. The infected portions were surface sterilized with 0.1% NaOCl for 1 min and washed thoroughly (at least thrice) with sterile distilled water. The surface sterilized parts were then cut into small species and finally transferred aseptically into sterile PDA slants. The slants were incubated at 28°±1C and were observed until sporulation (10–12 days). Sporulated cultures were subjected to microscopic studies following staining with cotton-blue in lactophenol as stated in section 3.4.1.2. The identity of the re-isolated fungi was confirmed by comparing it with the original fungal isolates. If an organism was consistently re-isolated then it was treated as a pathogen.

Similarly Koch's postulations were verified after inducing leaf infections in young pointed gourd plants. Inoculation was done by spraying the plants with conidial suspension (1 x $10^6$ conidia $ml^{-1}$) and the pathogen was re-isolated after 10 days from infected leaves.

In case of experiments with pointed gourd fruits, the fruits were inoculated by conidial suspension (1 x $10^6$ conidia $ml^{-1}$) prepared from 10–12 days old cultures of the fungal isolates. After 3–5 days of inoculation, when disease symptoms were evident on the fruits, infected portions were cut out with a sterile sharp blade and was transferred into sterile PDA slants following surface sterilization. The isolated pathogen was studied microscopically for its morphological features and spore characters. The culture characteristics in PDA were also recorded. The findings were compared with the original isolate. If the isolated fungi were similar to the original isolate, then it was considered as pathogen.

### 3.4. Identification of isolated fungal pathogens

In order to identify the isolated fungal pathogens, studies were undertaken on the phenotypic characters including colony characteristics and microscopic studies. Furthermore, phylogenetic characterization was also done based on ribosomal RNA genes (ITS and 28S rRNA genes) and Actin gene sequences (ACT) that were obtained following PCR amplification.

#### 3.4.1. Studies on phenotype

Phenotype studies included the colony morphology of each fungus and the morphology of their hyphae and conidia. Studies on cellular morphology were conducted under microscope. Culture morphology was studied by observing the culture characteristics in PDA plates.

##### 3.4.1.1. Colony characteristics

i. In order to study the colony characteristics of the isolated fungal pathogens, each fungus was inoculated centrally on sterile PDA plates (9 cm in diameter) and incubated for 2–3 days at 28°±1C for the colony to develop as mycelial mats.

ii. Subsequently, mycelial agar discs (5 mm in diameter) were cut with a sterile cork borer from the radially advancing zone of growing hyphae and each disc was placed at the centre on fresh sterile PDA plates.

iii. The plates were incubated at 28±1°C for 10 days for adequate sporulation.

iv. Different characteristic parameters of a fungal culture (colour and texture of mycelia; pattern of radial growth; colour, texture and quantity of spores etc.) were recorded at regular intervals.

**3.4.1.2. Microscopy**

i. A small portion of the mycelia from each sporulated pure fungal culture was placed on separate clean grease free glass slides and stained using cotton-blue in lactophenol.

ii. The slides were then mounted with cover glass, sealed and observed under light microscope at 40x (Leica Application Suite V4.4 microscope equipped with Leica MC 120 HD digital camera, Singapore).

iii. Detailed morphological properties of the fungi such as septation of hyphae, type and shape of spore, etc. were observed and recorded.

iv. Length and breadth of spores and breadth of mycelia were also measured (Amadi *et al.,* 2014).

**3.4.2. Studies of fungal genes for identification**

The isolated fungi were then identified by molecular techniques using polymerase chain reaction. Gene sequences that included partial 18S rRNA small subunit (SSU), complete internal transcribed spacer region 1 (ITS1), 5.8S rRNA gene, internal transcribed spacer region 2 (ITS2) and partial 28S rRNA large subunit (LSU) gene were analysed. Another gene sequence of D1/D2 region of 28S rRNA large subunit (LSU) was also used for confirmation of the isolates. This is an era of molecular techniques and for better understanding of the phylogeny of the isolated fungi, hence, Actin gene (ACT) was also isolated. The sequences were obtained by PCR amplification of the target genes using genomic DNA as the template. The universal forward and reverse primers were ITS1 and ITS4, which hybridizes with the end of the 18S rRNA gene and with the beginning of the 28S rRNA gene respectively (White *et al.*, 1990). The D1/D2 region of 28S rRNA large subunit (LSU) was amplified using NL1 and NL4 primers (O'Donnell 1993).

The Actin gene was amplified by using Act 1 and Act 2 primers (Bleve *et al.*, 2003). All the primers used have been presented in the table 3.2.

**3.4.2.1. Isolation of fungal genomic DNA**

Genomic DNA was extracted from fungal cultures following CTAB method (Dellaporta *et al.*, 1983) modified by Haible *et al.* (2006). CTAB-DNA extraction buffer was prepared by mixing 2% CTAB, Tris-100 mM [pH-8], 1.4M NaCl, 20 mM EDTA [pH-8] and 2µl/ml β-mercaptoethanol. Detailed step of CTAB method are as follows:

i. The fungi were inoculated in 50 ml PDB taken in 250 ml conical flasks for 15 days at 28±1°C. Mycelia were harvested by filtering through sterile muslin cloth, washed thrice with sterile distilled water, dried and ground to a fine powder in liquid nitrogen using sterile mortar and pestle.
ii. One gram of tissue was then homogenized with 5 ml pre-warmed (at 65°C) DNA extraction buffer.
iii. The resultant homogenate was transferred in a 1.5 ml micro centrifuge tube and incubated at 65°C for 1h, in a dry bath and the homogenate was mixed occasionally by gentle swirling.
iv. After incubation, homogenate was removed from the dry bath and 0.6 volume of chloroform-isoamyl alcohol (24:1) was added.
v. The homogenate was mixed thoroughly by inversion of the up side down and again down side up.
vi. After 15 minutes the mixture was centrifuged at 11,000 rpm for 10 minute and aqueous phase was transferred to another 1.5 ml micro centrifuge tube.
vii. 0.6 volume of isopropanol was added to the sample to precipitate the DNA.
viii. DNA was pelleted by centrifuging at 12,000 rpm in 1.5 ml centrifuge tubes for 15 min at 4°C.
ix. DNA was washed with 70% ethyl alcohol and dried overnight.
x. Dried DNA was dissolved in $T_{10}E_1$ buffer [pH-8].

### 3.4.2.2. RNAse treatment

The genomic DNA extracted by CTAB method contained RNA which was removed by RNase treatment.

i. 2.5 µl RNAse was added to 0.5 µl of crude DNA and mixed gently and incubated at 37°C for 1h.
ii. After this 0.4 ml of chloroform-isoamylalcohol (24:1) was added and was mixed thoroughly for 15 minutes. Finally, the resultant mixture was centrifuged for 15 min at 12,000 rpm.
iii. Supernatant was removed except the white interface layer and DNA was reprecipitated from the supernatant by mixing absolute alcohol (supernatant: Alcohol: : 1: 2).
iv. DNA was pelleted by centrifugation at 12,000 rpm for 15 min and then washed with 70% alcohol.
v. Dried DNA was then re-dissolved in $T_{10}E_1$ buffer [pH-8] and stored at -20°C for further use.

### 3.4.2.3. Quantification of DNA

Purity of DNA was estimated by computing the $OD_{260}/OD_{280}$ ratio. The absorbances were recorded at wavelengths of 260 nm and 280 nm. In the sample, the reading at 280 nm quantifies protein and the reading at 260 nm quantifies nucleic acid. For pure DNA, the $OD_{260}/OD_{280}$ value ranges from 1.8 to 2.0. The standard value of 1 OD at 260 nm corresponds to 50 ng/µl of dsDNA. For quantification of the DNA purified from fungi, the DNA sample (1 µl) was diluted in 49 µl TE buffer and absorbance was recorded at 260 and 280 nm in UV-Visible spectrophotometer (Varian, Australia).

### 3.4.2.4. Gel electrophoresis

The quality of purified genomic DNA was checked by agarose gel electrophoresis performed in a submarine gel electrophoresis system (Bangalore genei (India) Pvt. Ltd., India).

i. Agarose was suspended in 1X TAE buffer (1%) and melted in water bath until clear solution was obtained.
ii. Ethidium bromide (0.5 µg/ml) was added after cooling to about 50–60°C, mixed and poured into the gel casting tray.

iii. The gel was allowed to solidify and then it was completely submersed in electrophoresis tank containing 1X TAE running buffer.

iv. DNA samples (6 µl) mixed with gel loading buffer (2 µl) were loaded onto wells. Electrophoresis was run at 60 volt for 1 hour.

v. The gel was then removed from the tank and viewed under UV light in a UV transilluminator (Bangalore Genei (India) Pvt. Ltd, Bangalore, India).

vi. Bright fluorescent orange bands indicated the presence of DNA.

**3.4.2.5. PCR Amplification**

Polymerase chain reactions (PCR) were performed using a MJ Mini Personal Thermal Cycler (Bio-Rad) with the help of gene specific primer (Table. 3.2). Twenty-five microliter reaction mixture was prepared and 2 µl of genomic DNA was taken as template. The reaction mixtures contained the following components:

| | |
|---|---|
| Taq buffer (5X) | 5.0 µl |
| Taq DNA polymerase(5u/µl) | 0.13 µl |
| dNTP mix (10 mM each) | 1.0 µl |
| $MgCl_2$ (25 mM) | 1.5 µl |
| Forward primer | 1.0 µl |
| Reverse primer | 1.0 µl |
| Template (DNA) | 2.0 µl |
| Sterile water | 13.37 µl |

**Table. 3.2: List of primers used for PCR amplification**

| Primer name | Primer sequence | References |
|---|---|---|
| **ITS** | ITS1: 5'-TCCGTAGGTGAACCTGCGG -3'<br>ITS4: 5'-TCCTCCGCTTATTGATATGC -3' | White *et al.*, 1990 |
| **28S** | NL1: 5'-GCATATCAATAAGCGGAGGAAAAG -3'<br>NL4: 5'-GGTCCGTGTTTCAAGACGG -3' | O'Donnell, 1993 |
| **Actin** | ACT-1: 5'-GTATTGTTCTCGACTCTGGTGATGG-3'<br>ACT-2: 5'- TCTCAGGTGGTGCAACGACC-3' | Bleve *et al.*, 2003 |

### 3.4.2.6. Detection of PCR amplicons in agarose gel

The PCR amplicons of the fungal ribosomal RNA genes (both ITS and 28S large subunit) and Actin genes were resolved on 1% agarose gel containing ethidium bromide following methods described earlier (section 3.5.2.4.). The resolved amplicons were observed under UV light in a UV transilluminator. The molecular weight of PCR products was measured by using molecular weight markers (100 bp/500 bp ladder, Promega) run parallel to samples.

### 3.4.2.7. Purification and sequencing of PCR products

Purification of the PCR product was done using GeneiPure™ Quick PCR purification kit following the manufacturer's protocol.

i. At first, 5 volumes of Binding Buffer was added to 1 volume of PCR product and mixed well.
ii. Then the GeneiPure™ column was placed into a 2 ml collection tube and the sample was loaded and centrifuged at 11,000 rpm for 1 minute.
iii. The flow through was discarded and the GeneiPure™ column was placed back in the collection tube.
iv. The GeneiPure™ column was then washed with 500 µl of Wash Buffer I and centrifuged at 11,000 rpm for 1 minute.
v. Then the flow through was discarded and the GeneiPure™ column was placed back to the collection tube.
vi. After that, one volume of Wash Buffer II was diluted with four volume of absolute ethanol just before use.
vii. Again, the GeneiPure™ column was washed with 700 µl of diluted Wash Buffer II and centrifuged at 11,000 rpm for 1 minute.
viii. Then the flow through was discarded and the GeneiPure™ column was placed back to the collection tube.
ix. Again, it was centrifuged at 11,000 rpm for 2 minutes to remove the traces of Wash Buffer and the collection tube was discarded.
x. The GeneiPure™ column was opened and placed in fresh and sterile 1.5 ml vial and incubated at 70°C for 2 minutes to ensure complete removal of ethanol.

xi. For elution of DNA, 50 µl of pre-warmed (in dry bath at 70°C for 5 minutes) of Elution Buffer was added to the center of the column and incubated at room temperature for 1–2 min.

xii. The purified amplicons were sequenced at Chromas Biotech Pvt. Ltd. (Bangalore, India).

#### 3.4.2.8. Phylogenetic analysis

i. The partial ribosomal RNA gene sequences (both ITS and 28S large sub-unit) and the Actin gene sequences of the pathogenic fungal strains were compared with other related gene sequences available in GenBank databases using the BLAST search facility at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.,* 1997).

ii. After that, with the aligned sequences the nucleotide identity matrices were formed with SDT 1.2 (Muhire *et al.*, 2014).

iii. Phylogenetic trees were generated by neighbour-joining method (Saitou & Nei, 1997) through Kimura-2 parameter in MEGA 6.0 (Tamura *et al.*, 2013) following alignment with ClustalW 1.6 (Thompson *et al.*, 1994). Confidence in the tree topology was determined by bootstrap analysis using 1000 re-samplings of the sequences (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2013).

### 3.5. Studies on growth and physiology of the fungal pathogens

#### 3.5.1. Influence of different culture media on growth and sporulation

i. In order to evaluate the vegetative growth and sporulation of isolated pathogenic fungi in media, seven different media *i.e.* potato dextrose agar (PDA), oat meal agar (OMA), Czapek Dox agar (CDA), Richards's agar (RA), yeast extract mannitol agar (YEMA), malt extract agar (MEA) and pointed gourd dextrose agar (PGDA) media were used.

ii. Each media taken in 9 cm Petri plates was inoculated with 5 mm mycelial agar discs taken from advancing zones of mycelia of 10 day

old fungal cultures in PDA plates (Section 3.4.1.1). Experiments were performed with three replications.

iii. The inoculated Petri plates were incubated for 10 days at 28°±1C. Radial growths of mycelia were measured at 2 days intervals to assess the mycelial growth in different media. Colony diameters were measured in each plate and mean diameter was computed for each pathogen.

iv. Sporulation was graded as poor (+), fair (++), good (+++) and excellent (++++) by visual observation.

v. For estimation of sporulation, a small portion of mycelia was taken in microscopic slides, stained in cotton blue-lactophenol and observed under microscope (as stated in section 3.4.1.2).

**3.5.2. Influence of pH on growth of fungi**

i. To evaluate the optimum pH for the growth of the fungi, PDA medium was used as growth medium. Inoculums (mycelia disc) of test fungi (prepared from 10 day old cultures in PDA) were transferred into the PDA plates.

ii. The media were adjusted to different pH ranging from 3.5–7.5 by adding 1N HCl or 1N NaOH. The plates were incubated at 28±1°C.

iii. Radial growths of mycelia were measured at 2 days intervals to assess the mycelial growth. Colony diameters were measured in each plate. The experiments were done in three replications for each fungus and also for each pH value. The mean diameter was determined for each pathogen.

**3.5.3. Influence of temperature on growth of fungi**

i. To evaluate the optimum temperature for the growth of the fungi, PDA medium was used as growth medium.

ii. Inoculums of all test fungi prepared as mycelia agar discs from 10 day old cultures in PDA were transferred into the PDA plates. The plates were then incubated at different temperatures ranging from 23°C–33°C at intervals of 5°C.

iii. Radial growths of mycelia were measured at 2 days intervals to assess the mycelial growth. Colony diameters were measured in each plate.

The whole process was done in three replications for all test fungi and for each selected temperature. Finally, mean diameter was calculated for each pathogen.

### 3.6. Molecular detection of defense related gene

#### 3.6.1. Isolation of Resistance gene analog (RGA)

##### 3.6.1.1. Extraction of total RNA

Total RNA was extracted from the pointed gourd leaves following the method of Chomczynski and Mackey (1995). All the plastic and glass equipments used for this experiment were made RNase free by treating with DEPC for overnight and autoclaved. These were then dried in Hot Air Oven at 60°C before use.

i. Firstly, 100 mg of plant tissue was ground in liquid nitrogen using mortar and pestle.
ii. Then 1 ml of Trizol-S reagent was added and grinding was continued.
iii. The resultant mixture was transferred to 2 ml microcentrifuge tubes and allowed to stand for 5 minutes at room temperature.
iv. Chloroform (200 µl) was added to each tube and vortexed.
v. After 10 minutes, the tubes were centrifuged (13,000 rpm for 10 minutes at 4°C) and the upper aqueous phase was transferred into fresh microcentrifuge tubes.
vi. The tubes were vortexed for 10 seconds after addition of 0.6 volumes of isopropanol and allowed to stand at room temperature for 10 minutes.
vii. The tubes were re-centrifuged under similar conditions and the supernatant was discarded.
viii. The pelleted RNA was washed with ethanol (70%), dried in air and dissolved in 40 µl of sterile and DEPC treated RNase free distilled water.
ix. The RNA solution was finally stored at –20°C for further use.

##### 3.6.1.2. Quantification of RNA

In order to determine the concentration of RNA sample, its absorbance at 260 nm was measured and an absorbance of 1.0, when the path length was 1.0 cm, was equivalent to about 40 µg/ml of RNA. For pure RNA sample, the

$A_{260}/A_{280}$ ratio should be 2.0. A ratio below 2.0 indicates protein contamination. Besides, for pure RNA sample the $A_{260}/A_{280}$ ratio should be ≥2.0. A ratio quite lower than this indicates contamination by phenolate ion, thiocyanates and other organic compounds mostly used during RNA extraction and can interfere with subsequent steps in Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

#### 3.6.1.3. Agarose gel electrophoresis of extracted RNA

The quality of extracted RNA was assessed by electrophoresis on a denaturing agarose gel, because RNA often forms secondary structure via intra-molecular base pairing, and this prevents the RNA migrating strictly according to its size. Agarose gel was prepared as follows:

i. One gram agarose was mixed with 85 ml of RNAse water and heated until proper melting, and then allowed to cool to 60°C
ii. Then, 10 ml 10X MOPS buffer, and 5 ml 37% formaldehyde (12.3 M) was added
iii. The gel was then allowed to solidify
iv. RNA was then loaded onto the gel after mixing it with ethidium bromide and 2X RNA loading dye and electrophoresed at 60V for 1 hr
v. Visualize the gel under UV- Transilluminator (GeNei, Bengalore).

#### 3.6.1.4. RT-PCR (Reverse Transcriptase-PCR) from total RNA

Extraction of resistance genes was done from total RNA using One Step M-MuLV RT-PCR kit (Genei, Bangalore). Resistance gene specific degenerate primers NBS-F1 (5′-GGIGGIGTIGGIAAIACIAC-3′) and NBS-R1 (5′-IAGIGCIAGIGGIAGICC-3′) were used to amplify the resistance gene analogs present in the pointed gourd plant. After amplification the products were electrophoresed at 5V/cm through 1.2% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) buffer and visualized under UV transilluminator following ethidium bromide staining of the gel (70 min in 1 µg/ml ethidium bromide).

#### 3.6.1.5. Purification of PCR products

After obtaining the desired PCR products, they were purified using Wizard SV Gel and PCR Clean-Up System (Promega) following manufacturer's protocol (section 3.4.2.7).

#### 3.6.1.6. Cloning of PCR product

The PCR product with expected size were cloned into the pGEM®-T Easy Vector using pGME®-T easy cloning kit (Promega, USA) following manufactures protocol. The details cloning procedure are as follows:

**3.6.1.6.1. Preparation of competent cells**

Competent cells of *E. coli* (DH5α, Promega) for transformation were prepared following the method of Sambrook and Russel (2001) with some modification. Detailed method of component cells preparation is carrying out with following steps:

i. A single colony of DH5α cells were grown overnight at 37°C on 10 ml LB (Luria-Bertani) broth medium.
ii. 100 µl of overnight growing culture was inoculated into 5 ml of fresh LB broth.
iii. After that the culture was grown 2 to 4 hrs. (till O.D. reached 0.3 to 0.4 at 600 nm) at 37°C on a rotary shaker at 100 rpm.
iv. The growing culture was transferred to 1.5 ml micro centrifuge tube and was pelleted by centrifugation at 6,000 rpm for 10 min at 4°C and the supernatant was discarded.
v. 750 µl of solution-I (mixture of $MgCl_2$ and $CaCl_2$) was mixed with pelleted cells and the cells were re-suspended by gently mixing.
vi. The mixture was centrifuged at 5000 rpm for 7 minutes at 4°C and the supernatant was discarded.
vii. The pellet was re-suspended in 750 µl of 100 mM $CaCl_2$ solution by gently mixing and incubated on ice for 45 minutes.
viii. The cells pellet was again recovered from the mixture by centrifugation at 5000 rpm for 5 minutes at 4°C.
ix. The supernatant was discarded and the pellet was re-suspended in 500 µl of 100 mM $CaCl_2$ and stored at -20°C for overnight.

**3.6.1.6.2. Preparation of ligation mixture**

The ligation mixture was prepared by using pGEM®-T Easy Vector, purified PCR product and 2X rapid ligation buffer in a 0.5 ml PCR tubes. Tubes containing ligation mixture were incubated overnight at 4°C. The ligation mixtures containing the following components:

| | |
|---|---|
| 2X rapid ligation buffer | 5 µl |

| | |
|---|---|
| pGEM®-T Easy Vector | 1 µl |
| $T_4$ DNA Ligase | 1 µl |
| DNA (PCR product) | 2 µl |
| Deionized $H_2O$ | 1 µl |

**3.6.1.6.3. Transformation**

Previously prepared competent cells (as described in section 3.10.9.1.) were used for the transformation purpose. The detailed transformation procedures are as follows:

i. 2 µl of ligation mixture was added to the 100 µl competent cells and incubated in ice for 45 minutes.
ii. After ice incubation heat shock was given at 42°C for 90 sec.
iii. Then the tube was transferred directly to ice and incubated for 10 minutes.
iv. After ice incubation 300 µl LB broth was added to the tube and incubated at 37°C for 2 hrs with shaking at 200 rpm.
v. Centrifugation was done at 6000 rpm for 6 minutes at 4°C.
vi. After centrifugation 300 µl supernatant was discarded and the remaining one was gently mixed with the pellet.
vii. Then 100 µl transformed culture were spread onto LB agar plates containing ampicillin (50 mg/ml), IPTG (20%) and X-gal (4%).
viii. The plates were incubated overnight at 37°C.
ix. Positive transformed cells were selected by blue-white screening.

**3.6.1.6.4. Detection of positive clone by colony PCR**

After successful transformation, the positive transformed cells were further tested through boiling lysis followed by PCR. Each of the colonies was mixed with 200 µl sterile water in a 0.5 ml PCR tubes. The mixture was then boiled at 100°C for 10 minutes in water bath. After boiling the mixture was rapidly cooled down and centrifuged at 7000 rpm for 5 minutes at 4°C. The supernatant was used as template for PCR reaction. PCR reaction was carried out following the process as described in the section 3.4.2.5.

**3.6.1.7. Sequencing and phylohenetic analysis of cloned product**

The positive cloned products were sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore, India. The nucleotide (nt) sequence was aligned using

ClustalW 1.6 (Thompson *et al.*, 1994) and submitted in the GenBank after BLASTn analysis (Altschul *et al.*, 1997). The phylogenetic tree of the resistance gene analog was generated through neighbour-joining method with Kimura-2 parameter using MEGA 6.0 (Tamura *et al.*, 2013).

### 3.6.2. Isolation of defense enzyme related genes

#### 3.6.2.1. Extraction of total RNA

Total RNA was extracted from the pointed gourd leaves following the method of Chomczynski and Mackey (1995) as described in section 3.6.1.1.

#### 3.6.2.2. Agarose gel electrophoresis of extracted RNA

Agarose gel Electrophoresis was done at 5V/cm in 1% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) buffer for 60 min for visualization of RNA under UV- Transilluminator (GeNei, Bengalore).

#### 3.6.2.3. RT-PCR (Reverse Transcriptase-PCR) from total RNA

Extraction of defense enzyme related genes *viz.* phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (POX), poly-phenol oxidase (PPO) was done from total RNA using One Step M-MuLV RT-PCR kit (Genei, Bangalore). Five sets of gene specific degenerate primers were used (Table. 3.3) to amplify the defense enzyme related genes present in pointed gourd plant. After amplification the products were electrophoresed at 5V/cm through 1% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) buffer and visualized under UV-transilluminator following ethidium bromide staining of the gel (70 minute in 1 µg/ml ethidium bromide).

**Table. 3.3: List of primer used for PCR amplification**

| Primer name | Primer sequence |
|---|---|
| **PAL** | F: 5'-ACAACAATGGGTTGCCATCGAATC-3'<br>R: 5'-ACTTGGCTAACACTGTTCTTGACA-3' |
| **Chitinase** | F: 5'-GCCGCYTTYYTBGCKCARAC-3'<br>R: 5'-TGNGSNGTCATCCAYAACCA-3' |
| **β-1,3 glucanase** | F: 5'-TAYATHGCNGTWGGNAAYGA-3'<br>R: 5'-CCANCCRCTYTCNGAHACMAC-3' |
| **POX** | F: 5'-GAYTGCTTYGTYGATGGGTGYGATG-3'<br>R: 5'-ADDTTGCYCATCTTBAYCATRG-3' |
| **PPO** | F: 5'-CCDTTCTGGAAYTGGATTC-3'<br>R: 5'-CNGCNGAGTAGAAGTTSCCC-3' |
| **F-Forward primer; R- Reverse primer** | |

### 3.6.2.4. Purification of PCR products

After obtaining the desired PCR products, they were purified using Wizard SV Gel and PCR Clean-Up System (Promega) following manufacturer's protocol (section 3.4.2.7).

### 3.6.2.5. Cloning of PCR product

The PCR product with expected size were then cloned into the pGME®-T Easy Vector using pGEM®-T easy cloning kit (Promega, USA) following manufactures protocol. The details cloning procedure has been described in section 3.6.1.6.

### 3.6.2.6. Sequencing and phylogenetic analysis of cloned product

The positive cloned products were sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore, India. The nucleotide (nt) sequence was aligned using ClustalW 1.6 (Thompson *et al.*, 1994) and submitted in the GenBank after BLASTn analysis (Altschul *et al.*, 1997). Nucleotide identity was calculated using SDT v1.2 (Muhire *et al.*, 2014). The phylogenetic tree of the defense enzyme related genes were generated through neighbour-joining method with Kimura-2 parameter using MEGA 6.0 (Tamura *et al.*, 2013).

## 3.7. Preparation and application of inducer-chemicals on pointed gourd plants

i. One-month old pointed gourd plants (of variety Swarna alaukik) were taken in pots of 30 cm diameter.

ii. Seven different chemicals *viz.* α-Aminobutyric acid (AABA), β-aminobutyric acid (BABA), γ-amino butyric acid (GABA), 2,1,3-Benzothiadiazole (BTH), Salicylic acid (SA), Abscisic acid (ABA) and Hydrogen peroxide ($H_2O_2$) were used as inducers to elicit resistance in the plants.

iii. Each chemical was dissolved in sterile distilled water ($10^{-3}$ M) and were applied separately on lower leaves of one month old plants using hand sprayer leaving 3–4 top most leaves. The treated plants were labeled appropriately. The elicitors were augmented with Tween-20 before spraying in order to ensure adhering.

iv. One hour after treatment, treated lower leaves were challenge inoculated with conidial suspension ($1x10^5$ conidia/ml) *Fusarium equiseti* (one of the pathogen of present study).

v. The entire experiment was performed with appropriate controls in four sets (untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated) for each pathogen and for each inducer. Plants were maintained in green house in a sterile environment under normal daylight condition throughout the experiment to avoid contamination.

vi. The upper leaves of untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated were harvested for studying expression of defense related enzymes after 0 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr, 5 day and 7 day following inoculation by the pathogens. Disease index was computed for the inoculated plants. The whole experiment was repeated thrice and the data were averaged.

### 3.8. Extraction and estimation of defense related enzymes

#### 3.8.1. Phenylalanine ammonia lyase (EC 4.3.1.5)

i. PAL activity was determined at the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Sadasivam and Manickam (1996).

ii. One gram of fresh leaves was dipped in liquid nitrogen for 10 minutes and crushed in 5 ml of 0.25 M borate buffer (pH-8.7) at 4°C.

iii. Filter through 4 layered muslin cloth.

iv. Centrifuge the filtrate at 12,000 g for 15 minutes at 4°C.

v. Yellowish green supernatant was used as crude enzyme.

vi. Then, 0.5ml of borate buffer, 0.25 ml of crude enzyme, 1.5 ml of distilled water and 1 ml of 0.1M L-phenylalanine were mixed and incubated for 30 minutes at 30°C.

vii. After that, the reaction was stopped by adding 0.5 ml of 1M Trichloroacetic acid.

viii. Absorbance values were recorded at 290 nm in UV-VIS spectrophotometer.

ix. Enzyme activity was expressed as μmol $min^{-1}g^{-1}$ fresh weight tissue using trans-cinnamic acid as standard.

$$\text{Phenylalanine ammonia-lyase activity} = \frac{\text{Concentration} \times \text{dilution factor} \times \text{final volume} \times 3}{\text{Initial weight} \times \text{volume of enzyme} \times 60}$$

### 3.8.2. Peroxidase (EC 1.11.1.7)

Peroxidase activity was determined according to procedure given by Hammerschmidt *et al.,* 1982.

i. One gram of fresh leaves was dipped in liquid nitrogen for 10 minutes and crush in 0.1M sodium phosphate buffer (pH-6.5) at 4°C.
ii. Then the homogenate was filtered through four layered muslin cloth and centrifuge the filtrate at 6000 g at 4°C for 15 minutes.
iii. Supernatant was collected and considered as crude enzyme.
iv. After that, 1.5 ml of 0.05M guaiacol and 200 μl of extracted crude enzyme were taken in a cuvette.
v. The cuvette was then placed in a UV-VIS spectrophotometer and initial reading was adjusted to zero at 420 nm.
vi. 100 μl of hydrogen peroxide (1%v/v) was added in cuvette and the changes in absorbance values were recorded for 5 minutes in 1 minute interval.
vii. The enzyme activity was expressed in unit enzyme activity. Change in absorbance [$\Delta A_{420}$ $min^{-1}g^{-1}$fresh weight tissue] of 0.001 was considered as unit of enzyme activity.

$$\text{Peroxidase activity} = \frac{\text{Mean O.D.} \times \text{Final volume} \times 10 \times \text{dilution factor}}{\text{Initial weight}}$$

### 3.8.3. β-1, 3-glucanase (EC 3.2.1.6)

Colorimetric method of Pan *et al.* (1991) was used for the assay of β-1, 3-glucanase enzyme, this method is also named as laminarin -dinitrosalicylate method.

i. One gram of fresh leaves were dipped in liquid nitrogen for 10 minutes and crushed in 5 ml of 0.05M sodium acetated buffer (pH-5.0) at 4°C.
ii. Filter through 4 layered muslin cloth.
iii. Centrifuge filtrate at 10,000 g for 15 minutes at 4°C.
iv. Supernatant was taken as crude enzyme.

v. Then, 15.6 µl crude enzyme and 15.6 µl of 4% laminarin was incubated at 40°C for 10 minutes.
vi. The reaction was stopped by adding 94 µl of dinitrosalicylic acid followed by heating for 5 minutes on boiling water bath.
vii. The final colour of the solution was diluted with 1 ml distilled water.
viii. Absorbance was taken in UV-VIS spectrophotometer at 500 nm.
ix. Enzyme activity was expressed in fresh weight basis (nmol $min^{-1}mg^{-1}$) using D-glucose as standard.

$$\text{β-1,3-glucanase activity} = \frac{\text{Concentration from graph} \times \text{final volume} \times \text{dilution factor}}{\text{Initial weight} \times 10}$$

### 3.8.4. Chitinase (EC 3.2.1.14)

Chitinase activity was determined according to the procedure described by Mahadevan and Sridhar (1982) with some modifications.

i. Pointed gourd leaves (1 g) were dipped in liquid nitrogen immediately after harvesting.
ii. The frozen leaves were crushed after 10 min in 5 ml of 0.5 M sodium acetate buffer (pH 5.2) containing 700 mg of PVP in a chilled mortar and pestle at 4°C.
iii. Filtered through four-layered muslin cloth.
iv. The filtrate was centrifuged at 10,000 g at 4°C for 15 min and the supernatant was used as crude enzyme source.
v. The assay mixture contained 0.5 ml crude enzyme extract, 1 ml colloidal chitin (1.8 mg/ml) and 0.25 ml of 0.1M sodium acetate buffer.
vi. The mixture was incubated at 37°C for 2 h.
vii. Distilled water (1 ml) was added to 1 ml of reaction mixture and boiled for 10 min in a water bath and subsequently centrifuged at 5,000 g for 3 min.
viii. The supernatant (0.5 ml) was added to 0.1ml of 0.8 M Potassium tetraborate, boiled exactly for 3 min on a water bath and cooled.

ix. Thereafter, 3 ml of p-dimethyl amino benzaldehyde (DMAB) reagent was added and incubated at 37°C for 20 min.

x. Absorbances (at 585 nm) were recorded immediately after incubation, in a UV-VIS Spectrophotometer (Systronics, Model no.118, India).

xi. Enzyme activity was expressed as mg GlcNAc $g^{-1}$ fresh weight tissue $h^{-1}$.

### 3.8.5. Poly-phenol oxidase (EC 1.14.18.1)

Poly-phenol oxidase enzyme assay was done following the method of Sadasivam and Manickam (1996).

i. One gram of fresh sample was dipped in liquid nitrogen for 10 minutes and crushed in 5ml of 50mM Tris-HCl buffer (pH-7.2) containing 0.4M sorbitol and 1.0 mM NaCl.

ii. Centrifuge homogenate at 12,000 g at 4°C for 10 minutes.

iii. Take supernatant and is considered as crude enzyme.

iv. After that, 2.5 ml of 0.1M sodium phosphate buffer (pH-6.5) and 0.2ml of crude enzyme was mixed in a cuvette.

v. The cuvette was placed in UV-VIS spectrophotometer and initial reading was adjusted to zero at 495 nm.

vi. Then, 0.3 ml of 0.01M catechol was added to reaction mixture cuvette and changes in absorbance were recorded at 1 minute interval upto 5 minutes.

vii. The enzyme activity was expressed as change in absorbance [Enzyme activity = K*($\Delta A min^{-1}$)µmol $min^{-1} g^{-1}$ fresh weight tissue (K=0.272 for PPO).

$$\text{Polyphenol oxidase activity} = \frac{\text{K}\times\Delta\text{O.D.}\times\text{Final volume}\times\text{dilution factor}\times 10}{\text{Initial weight}\times 1\text{ minute}}$$

*where K= 0272 for polyphenol oxidase

## 3.9. Quantification of transcripts of defense related genes from pointed gourd plants by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

### 3.9.1. Preparation and application of inducer-chemicals on pointed gourd plants

i. One-month old pointed gourd plants (of variety Swarna alaukik) were taken in pots of 30 cm diameter.

ii. Seven different chemicals *viz.* AABA, BABA, GABA, BTH, SA, ABA and $H_2O_2$ were used as inducers to elicit resistance in the plants.

iii. Each chemical was dissolved in sterile distilled water ($10^{-3}$ M) and were applied separately on lower leaves of one month old plants using hand sprayer leaving 3–4 top most leaves. The treated plants were labeled appropriately. The elicitors were augmented with Tween-20 before spraying in order to ensure adhering.

iv. The entire experiment was performed with appropriate controls in two sets ('chemical inducer treated' and 'untreated control plants') for each inducer. Plants were maintained in green house in a sterile environment under normal daylight condition throughout the experiment to avoid contamination.

v. The upper leaves of 'treated' and 'untreated-control' were harvested for studying expression of defense related genes after 72 hr of post-treatment.

**3.9.2. Extraction of total RNA**

Total RNA was extracted from the leaves of inducer (AABA, BABA, GABA, BTH, SA, ABA and $H_2O_2$) sprayed pointed gourd plants and 'untreated control' plants following the method of Chomczynski and Mackey (1995) described in section 3.6.1.1.

**3.9.3. Quantification of RNA**

Extracted RNA was then quantified by taken absorbances at wavelengths of 260 nm. The standard value of 1 OD at 260 nm corresponds to 40 ng/µl of ssRNA. For quantification of the RNA purified from the 'treated' and 'untreated control' plants, the RNA sample (10 µl) was diluted in 490 µl nuclease free water and absorbance was recorded at 260 nm in UV-Visible spectrophotometer (Varian, Australia). The RNA yield was measured.

Concentration of RNA sample = 40 X $A_{260}$ X dilution factor

**3.9.4. Agarose gel electrophoresis of extracted RNA**

Agarose gel Electrophoresis was done at 5V/cm in 1% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) buffer for 60 min for visualization of RNA under UV- Transilluminator (GeNei, Bengalore).

### 3.9.5. Isolation of housekeeping gene (26S rDNA) as a reference gene for expression study

The 26S rDNA gene was used as a reference gene for the expression analysis. The gene was isolated by extracting the RNA from pointed gourd plant as suggested in the section 3.9.2 and amplified by RT-PCR using specific primers (F: CACAATGATAGGAAGAGCCGAC and R: CAAGGGAACGGGCTTGGCAGAATC). For amplification one Step M-MuLV RT-PCR kit was used (Genei, Bangalore) following the manufacturer's protocol (section 3.6.1.4). The amplified products were sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore, India. The nucleotide (nt) sequence was aligned using ClustalW 1.6 (Thompson *et al.*, 1994) and submitted in the GenBank (Acc. No- MN176624) after BLASTn analysis (Altschul *et al.*, 1997).

### 3.9.6. RT-PCR (Reverse Transcriptase-PCR) from total RNA

For the semi-quantitative analysis, Resistance gene analog (PGRGA), PAL, peroxidase, chitinase, β-1,3-glucanase, PPO and 26S rDNA genes were amplified from the extracted RNA of inducer treated plants and untreated control plants using One Step M-MuLV RT-PCR kit (Genei, Bangalore) following the manufacturer's protocol (section 3.6.1.4). Gene specific degenerate primers were used (table 3.4) for the amplification.

**Table. 3.4: List of primer used for Reverse Transcriptase PCR amplification**

| Primer name | Primer sequence |
|---|---|
| **PGRGA** | F: 5′-GGIGGIGTIGGIAAIACIAC-3′ |
| | R: 5′-IAGIGCIAGIGGIAGICC-3′ |
| **PAL** | F: 5'-ACAACAATGGGTTGCCATCGAATC-3' |
| | R: 5'-ACTTGGCTAACACTGTTCTTGACA-3' |
| **Chitinase** | F: 5'-GCCGCYTTYYTBGCKCARAC-3' |
| | R: 5'-TGNGSNGTCATCCAYAACCA-3' |
| **β-1,3-Glucanase** | F: 5'-TAYATHGCNGTWGGNAAYGA-3' |
| | R: 5'-CCANCCRCTYTCNGAHACMAC-3' |

| Primer name | Primer sequence |
|---|---|
| **POX** | F: 5'-GAYTGCTTYGTYGATGGGTGYGATG-3' |
| | R: 5'-ADDTTGCYCATCTTBAYCATRG-3' |
| **PPO** | F: 5'-CCDTTCTGGAAYTGGATTC-3' |
| | R: 5'-CNGCNGAGTAGAAGTTSCCC-3' |
| **26S rDNA** | F: CACAATGATAGGAAGAGCCGAC |
| | R: CAAGGGAACGGGCTTGGCAGAATC |

**F-Forward primer; R- Reverse primer**

### 3.9.7. Agarose gel electrophoresis of amplified defense related gene product

After amplification the products were electrophoresed at 5V/cm through 1.2% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) buffer and visualized under UV transilluminator following ethidium bromide staining of the gel (70 min in 1 µg/ml ethidium bromide).

### 3.9.8. Digital analysis of Reverse Transcriptase PCR electrophoresis gel

The gel images of the amplicons (RT-PCR bands) were captured under ultraviolet light by camera (Canon EOS 3000D DSLR, Taiwan). All the experimental conditions and parameters used were kept constant throughout the study. The image was then saved (in tiff. format) on a computer for digital image analysis using ImageJ software version 1.5.2v (Schneider *et al.*, 2012).

**3.9.9. Statistical analysis:** Statistical analysis (Standard deviation and Standard error) was done by using Microsoft Office Excel 2007.