Chapter-3

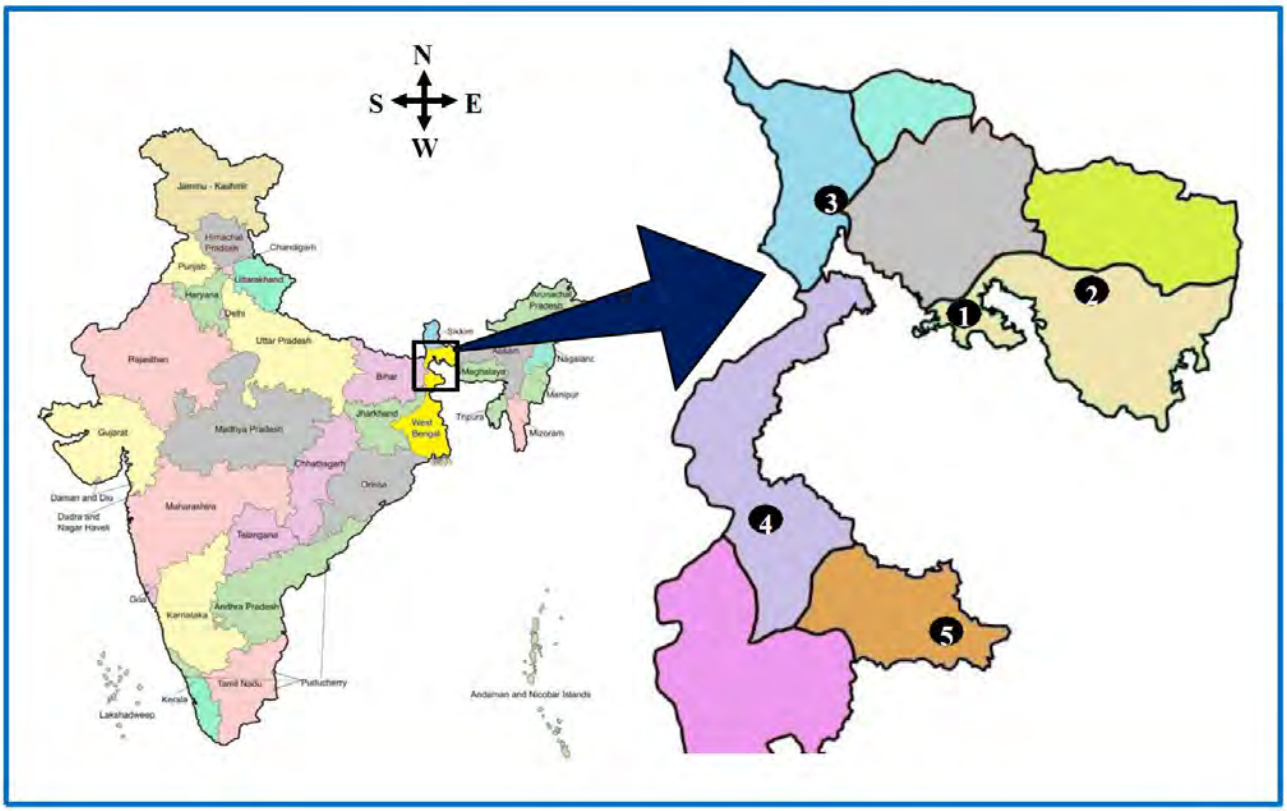
MATERIALS AND METHODS

3.1: Survey of different tomato growing fields of North Bengal, sample collection and selection of sample collection fields:

Roving survey was carried out in the different tomato growing fields of North Bengal to identify the affected areas (Fig.3.1). Severely infected areas were identified and affected plants were collected. Soil samples were collected from the rhizosphere of the infected plants and were brought to the laboratory of Department of Botany, University of North Bengal for further experiments. Wilted tomato plants were collected and were brought to the laboratory. Fifty different collection points were identified from where diseased plants were collected. Out of the 50 different collection points 26 points were found to be bacterial wilt prone area. The samples from these 26 points were subjected to bacterial isolation by ooze collection procedure. Thus all the 26 bacteria isolated were subjected to preliminary identification by morphology and biochemical characteristics. However, the most virulent isolates were also identified by molecular 16S rRNA studies.

3.2: Host plants:

One local variety and four certified tomato seed varieties (PKM-1, Vaishali, Rupali and Rashmi) were used during the present study. All the five varieties were selected for plantation in the experimental garden, Department of Botany, University of North Bengal. The four certified varieties were selected on the basis of their availability in the local seed shops and also for their popularity among the local farmers. On the basis of verbal discussion with the local farmers it was known that they preferred the varieties on the basis of their satisfactory growth in the agro-climatic condition of North Bengal. Excluding tomato plants, the brinjal and potato plants were also used for the host range study. The varieties of brinjal potato and Chilli were 'Muktakesi', 'Kufri jyoti' and 'Kull Sona" respectively. About 10 cm long healthy plants were collected from farmers' fields of Phansidewa (District Darjeeling)



1. **Haldibari[26.33°N88.77°E](Coochbehar)**
2. **Ghoshkadanga[26.43°N89.27°E](Coochbehar)**
3. **Siliguri[26.71°N88.43°E](Darjeeling)**
4. **Durgapur[25.52°N88.15°E](UttarDinajpur)**
5. **Balurghat[25.24°N88.79°E](DakshinDinajpur)**

Figure:3.1:Mapshowingplacesofdiseasedsamplecollection.Nameinparenthesesa redifferentdistricts ofsub-Himalayan,WestBengal.

3.3: Plant germination and maintenance:

Tomato seeds of the selected varieties were surface sterilized with 0.1% mercuric chloride solution for 1 min and then washed thrice with sterile distilled water. Seeds were dipped in seed-bed (earthen tray or directly on soil) for germination. Tomato Seedlings about 10 cm long, from the earthen tray were finally transferred to earthen pots (16 cm in diameter and 12 cm in height) containing soil-farm yard manure mixture of 200g of farm yard manure and 1800g of fine soil. The same mixture was also prepared and used for plants transferred to plots. One plant was grown in one pot. In the plots plants were sown in a row in such a manner so that distance between plant to plant and row to row was 45 cm (Fig. 3.2).

Other plants (Potato, Brinjal and Chilli) were also transferred to experimental pots/plots directly from farmer's fields. The plants were maintained in the garden at natural temperature (25^0C-30^0C) and light; pH of soil was about 5.6 to 6.0. Plants were watered as and when required. The source of water was natural tap water. Weeds were removed regularly.

3.4: Bacterial pathogens/antagonist: The infected plant samples and rhizosphere soil samples were collected and brought to the laboratory for the isolation of the bacteria through bacterial ooze collection (in case of pathogen) and by serial dilution technique as suggested by Dhingra and Sinclair (1995) in case of isolation of antagonistic soil bacteria.

3.4.1: Isolation of pathogenic bacteria isolates from infected plants: All plant samples were washed with sterile distilled water and surface of the crown region was surface sterilized with 70% alcohol. Finally, a cross section of the plant stem was made with a sharp sterilized blade. The root portion was removed and the cut end of the stem was immediately introduced in to a tube containing sterile distilled water. A stream of white, milky layer of bacterial cells (bacterial ooze) in clear sterile distilled water was observed. Then the water was used as sample for isolation of bacteria as suggested by Leppla *et al.*

(2004). Bacterial suspension (0.1 ml) was spread on *Pseudomonas solanacearum* agar medium and incubated at 32° C for 24 hours.

Fig. 3.2: a) Tomato plants in the experimental garden of Department of Botany; b) Potted plants in the experimental garden

3.4.2: Isolation of antagonistic bacteria from soil:

The rhizosphere soil samples were semi dried in laboratory and then 10 gram soil was mixed in 100 ml of sterile distilled water. The resultant solution was considered as stock solution. From the stock solution serial dilution series were made up to the dilution of 10^{-5}. The 10^{-4} dilution has given the ideal plate count (30 cfu to 300 cfu per ml) count. One ml of this dilution was allowed to grow in 20 ml Nutrient Agar medium supplemented with 2mg Nystatin (Fungi static compound). After 72 hours of incubation at 30°C, a large bacterial colony intermingled with several other colonies. If any bacterium found to show antagonistic behaviour against other bacteria nearby in the plate, then that bacterium was picked up by sterile tooth-pick and plated in a sterile Nutrient Agar medium. Thus antagonistic bacteria were isolated and purified.

In the present study one fungal antagonist *Trichoderma harzianum* was used which was procured from Prof. Aniruddha Saha of Molecular Plant pathology Laboratory of Department of Botany, Universiyty of North Bengal. The fungal culture was originally collected from Indian Agricultural research Institute, New Delhi (ITCC-4572).

3.5: Pathogenicity test:

Pathogenicity test was performed in potted plants as well as in field grown tomato plants.

3.5.1: Pathogenicity test in potted plants and verification of Koch's postulations:

Bacterial pathogens of the present study were subjected to pathogenicity test following the method as suggested by Hoque and Mansfield, 2005. Healthy (10 cm long) tomato plants [Local cultivated variety] were transplanted into earthen pots (size: 16 cm diameter × 12 cm in height) containing sterilized garden soil. All the bacterial isolates to be tested were freshly cultured in nutrient broth medium. Ten ml of 72 h old broth was diluted to 50 ml by

sterilized distilled water. The diluted culture was mixed with the soil surrounding the test plant. In case of control 50 ml of sterilized distilled water was added instead of diluted culture. Incidence of *Ralstonia wilt disease* was evaluated up to 15 days at 3 days intervals and was recorded as days after pathogen inoculation (dapi). Severity of the symptoms was graded into five disease classes as suggested by Kobriger et al., (1998) with minor modifications. Wilting index was graded as follows: + = up to 20% wilted; ++ = 20-40% wilted; +++ = 40-60% wilted and++++ = 60-80% wilted and +++++= 80-100% wilted. Ten plants were taken for each treatment. Average disease development of ten plants of each treatment was considered as wilting index to evaluate disease development.

Koch's postulations were verified in order to ascertain the isolated bacterium is a pathogen or not. For this, each bacterium, isolated from bacterial ooze was tested for its ability to induce wilt disease in tomato plants grown in pots and those have proved potential as pathogen in 'pathogenicity test' were subjected to re-isolation of the bacteria from the cut end of the stem as mentioned earlier. The re-isolated bacterium of each such isolation was biochemically identified as *Ralstonia solanacearum*.

3.5.2: Pathogenicity test in field grown whole plants and disease assessement in susceptible variety:

Soil was ploughed and solarized for seven days thereafter healthy tomato plants (10-12 cm long) of variety PKM-1 were sown in the experimental garden. Distance between plant to plant and row to row was 45 cm. The plants were maintained by natural tap water. Bacterial isolates to be tested were freshly cultured in nutrient broth medium. Ten ml of 72 h old broth was diluted to 50 ml by sterilized distilled water. The diluted culture was mixed with the soil surrounding roots of the test plants. In case of control 50 ml of sterilized distilled water was added instead of diluted culture. Control plants and

experimental plants were kept at 4 meter distance (Fig. 3.3). Incidence of disease was evaluated as described in section 3.5.1.

3.6: Morphological and Biochemical characterization of 26 bacterial isolates:

The classical approach to bacterial identification involves preliminarily microscopic examination following Gram reaction which divides bacteria into two broad groups (Gram + ve and Gram – ve). In addition several biochemical tests were performed to identify the bacteria up to the genus level as suggested by Trigiano *et al.* (2004). The major morphological and biochemical experiments performed were motility test, Gram staining, suitable growth condition (aerobic/anaerobic) on common laboratory media (Nutrient agar & nutrient broth), Yellow pigmentation on YDC medium, Growth on D1M medium, Growth above 40°C, Growth bellow 4°C, Oxidase test. Finally different carbohydrate utilization test with KB009 HiCarbohydrate™ Kit were done for some selected bacterial pathogens.

3.6.1: Gram staining:

Test bacterium was grown in Nutrient Agar slant for 24 hour. Loop full of bacteria was placed on a clean grease free slide and smear was made. The smears were dried in room temperature and air dried smear was heat-fixed. Then the slides were placed in a tray and flooded with crystal violet [composition: 2g Crystal violet, 20 ml alcohol (95%), 80 ml Ammonium oxalate solution(1%)]. Stained for 1 minute and washed in running tap water for 3 seconds. Then the slide was flooded with Burke's Iodine [1 g Iodine, 2g Potassium Iodide, and distilled water 1000 ml] and allowed to stain for 1 minute. Slides were washed with 95% ethanol, which was poured drop by drop holding the slides in slanting condition against a white background till no colour came out from the lower portion of the slide. Thereafter, the saffranin solution was poured on the smears. After 1 minute of staining the slide was

rinsed with water, air dried and was ready for light microscopic study. (Bartholomew, 1962)

Fig. 3.3: Pathogencity test: a) Healthy polant (uninoculated) b)Plant .showing wilt symptom following inoculation by *Ralstonia solanaccarum* (isolate RSG-01)

3.6.2: Aerobic/anaerobic growth study:

Ralstonia solanacearum isolates were grown in thioglycolate broth. Where aerobic bacteria cannot grow and anaerobic bacteria grow at the base because anaerobes are poisoned by oxygen as the concentration of oxygen is lowest at the base. Sodium thioglycolate in the broth consumes oxygen and allows the growth of obligate anaerobes.

3.6.3: Yellow Pigmentation study in YDC medium:

After preparation of YDC medium plates, the plates were inoculated by all the 26 *Ralstonia solanacearum* isolates. If yellow pigmentation was found, then that bacteria were marked as YDC positive.

- **Composition and preparation of YDC medium:**

Yeast extract	10 g
Dextrose	20 g
$CaCO_3$ (light powder)	20 g
Agar agar	20 g
Distilled water	1000ml

Dextrose was separately autoclaved at 15 lb p.s.i. for 15 minutes at 121°C in half of the distilled water and other ingredients were also autoclaved in the other half of the distilled water at 15 lb p.s.i. for 15 minutes at 121°C. Finally solutions were combined and stirred before pouring plates on that CaCO3 was evenly distributed (Schaad, 1988).

3.6.4: Growth on D1M Medium:

Growth on D1M medium was studied for all the 26 bacteria. Fresh D1M plates were inoculated by all the 26 pathogenic bacteria. Bacterial plates were

incubated at 30°C. If any growth was observed visually, then it was recorded as positive. If no growth was found then it was recorded as negative.

- ***Composition and preparation of D1M Medium*:**

Cellobiose	5.0g
NH_4Cl	1.0g
NaH_2PO_4	1.0g
KH2PO4	1.0g
MgSO4, 7H2O	3.0g
Malachite green	3.0 g
Agar agar	15g
Distilled water	1000ml

All the ingredients were mixed at proper ratio and pH was adjusted at 7.0 and then autoclaved at 15 lb p.s.i. for 15 minutes at 121°C. After autoclave the media was cooled at 50 to 55°C and then poured into Petri plates (Schaad, 2001).

3.6.5: Oxidase test:

This test was performed to identify the suspected isolates containing the Cytochrome Oxidase which reduces the electrons of electron transport chain of the isolates to water. When the electron donor was oxidized by Cytochrome Oxidase it turns a dark purple colour which was confirmed the positive test. Bacterial cultures (24 h old) were smeared across a filter paper moistened with freshly prepared 1% tetramethyl-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 seconds indicated a positive reaction (Cowan and Steel, 1993).

3.6.6: Catalase test:

The test organisms were inoculated in a nutrient agar plate. the plate was incubated in a slop for 24 hour. One ml of Hydrogen peroxide solution (3%) was added down the slop. Immediately if gas evolved then that is the indication of catalase activity (Cowan and Steel, 1993).

3.6.7: Carbohydrate utilization test:

For the carbohydrate utilization test the KB009 HiCarbohydrate™ Kit of HIMEDIA was used to study a biochemical profile of the suspected isolates. In this kit the tests are based on the principle of pH change and substrate utilization. After incubation, the metabolic changes were occurred of the isolates and it was indicated by the spontaneous colour change in the media. The kit was divided into three parts like Part A (KB009A), Part B1 (KB009B1) and Part C (KB009C). The pure cultures were used for the test. Homogenous suspension was prepared for the individual isolates with sterile saline and the density of the suspension was 0.5 OD at 620nm. 50µl inoculum was added by surface inoculation method for each selected isolate and incubated at 35°C for 24 to 48 hours.

3.6.8: Growth Below 4ºC and above 40ºC:

Each test bacterium (pathogenic or antagonistic) was freshly streaked on three Nutrient agar medium plates. In case of pathogenic bacteria one plate was incubated at 4°C, one plate was incubated 40°C and the third plate was incubated at 30°C (Control). Growth of the bacterial streak was observed against the control bacterial plate kept at 30°C. Plates were observed up to 72 h. If no growth was found on the basis of visual observation the bacterium growth was recorded as negative. The antagonists grown in nutrient agar medium were incubated at 25°C, 30°C, 37°C and 42°C. Plates were observed up to 72°C.

3.6.9: Host range test of bacterial pathogen:

Generally bacteria survive in a wide host range and cause disease in most preferable target host. The host range test of *Ralstonia solanacearum* was performed in three different cultivated plants belong the Solanaceae family. The test was performed by inoculation of the *Ralstonia solanacearum* in soil of experimental pots as mentioned in case of pathogenicity test. The symptoms were observed for a period of time up to 15 days at 3 days intervals. The hosts which showed symptoms were recorded.

3.7: Morphological study:

3.7.1: Shape of the cells:

To examine the shape and size of cells, a drop of cell suspension of the test organism was placed on a clean grease-free slide, air dried and stained with 10 times diluted carbol fuchsin (phenol 85g, basic fuchsin 15 g, ethanol 250 ml, distilled water 1250ml) and was observed under microscope. Diameter was measured with a standard occular micrometer.

3.7.2: Motility test:

Motility of the isolated bacteria was detected in semisolid motility medium (Barow and Feltham, 1993). Stab containing motility medium was inoculated by a straight inoculating needle. The tubes were incubated at 30°C for 48h and observed for diffused growth around the line of inoculation which indicate positive motility of the test bacteria.

- **Modified motility medium (Hajna, 1950) composition & procedure:**

Peptone	10g
Beef extract	3g
NaCl	5g
Agar	4g
Gelatin	80g
Cystein	0.2g

Ferrous ammonium sulphate	0.2g
Sodium citrate	2g
Distilled water	1000ml

Gelatin was soaked in water for 30 minute. Other ingredients were added and heated to dissolve. Finally, the medium was sterilized at 15lb pressure for 20 minutes.

3.7.3: Light Microscopy:

Gram stained smears on slides as described in section 3.6.1 was observed under light microscope at 40x (Leica Application Suite V4.4 microscope equipped with Leica MC 120 HD digital camera, Singapore). The bacteria tested were determined whether it was Gram positive or Gram negative. Gram negative bacteria show pink or light red colour whereas Gram positive bacteria show blue colour under light microscope.

3.7.4: Scanning Electron Microscopy:

One sterile cover glass was placed in a sterile Petriplate and a bacterial smear was prepared on that coverglass. The cover glass was subjected to series of treatment prior to observation underscanning electron microscope (SEM). The process of Samaranayake *et al.* (2005) was followed for treating the bacterial smear on cover glass. The bacteria were fixed with 2.5% glutaraldehyde solution for one hour. Glutaraldehyde was removed by decanting. Then dehydration of the material was done by an ascending series of ethanol. After dehydration the samples were coated with gold (IB2-ion coater, Japan) and observed under scanning electron microscope [Model: Hitachi S-530(Japan) 1986].

3.8. Studies of bacterial genes for identification:

The isolated bacteria were then identified by molecular techniques using polymerase chain reaction. Gene sequences that included 16S rRNA were analyzed. Molecular detection is the most specific technique for identification of the isolated bacteria. It is also required for better understanding of the phylogeny of the isolated bacteria. In addition, another gene sequence of *fliC* gene was also used for confirmation of the isolates following BLASTn analysis.

3.8.1: DNA isolation:

In this study the bacterial genomic DNA were isolated following the process of Ausubel *et al.*, (1992). At the beginning of the DNA isolation process the Nutrient Broth culture media was prepared for the preparation of fresh bacterial culture. Incubation of 1 cfu in 5 ml of Nutrient Broth was done at 30°C for overnight at 120 rpm. After getting the sufficient growth the cell suspension was centrifuged for 2 min at 13,000 x g. Then the supernatant was discarded and the pellet was first dry and again resuspended in 567 ml of TE buffer. Then the suspension of 30 ml of 10% SDS and 3 ml of proteinase K (20 mg mL-1) was mixed uniformly and incubated at 37°C for one and half hours. After completion of the incubation the 100 ul of 5 mol L^{-1} NaCl and 80 ml of CTAB buffer containing 10 g CTAB and 4.1 g NaCl in 100 ml of water was added and again incubated at 65°C for 10 minutes in water bath. Finally after deproteination centrifugation was done at 10000 x g and after discarding the supernatant, the pallet was mixed with double volume of isopropanol (70%). Again centrifugation was done at 10000 x g for 10 minntes at 4°C. Finally the supernatant was discarded and the pellet was resuspended in 20µ TE buffer and stored at -20°C.

3.8.2: PCR technique:

Polymerase chain reactions (PCR) were performed using a MJ Mini Personal Thermal Cycler (Bio-Rad) with the help of gene specific primer. A set of 16S

rRNA primer as suggested by Boudazin *et al.* (1999) and a set of primer named Rsol_*fliC* suggested by Sconfeld et al. (2003) for identification of *Ralstonia solanacearum* were used. Twenty-five microliter reaction mixture was prepared and 2 µl of genomic DNA was taken as template. Amplicons were analyzed by electrophoresis in 1% agarose gel. Finally the amplicons were cloned.

- **List of primers used for PCR amplification**

Primer	Primer sequence	Reference
16S rRNA	Forword 5' – GAGTTTGATCATCATCGCTCAG – 3' Reverse 5' – GGCGGGACTTAACCAACAT – 3'	Boudazin *et al.* (1999)
Rsol_*fliC*	Forword 5' – GAACGCCAACGGTGCGAACT – 3' Reverse 5' – GGCGGCCTTCAGGGAGGTC – 3'	Sconfeld et al. (2003)

- **PCR reaction mixture for 16S rRNA**

Go-Taq-Green Mastermix (2X)	12.5 µl
Forward primer	0.5µl
Reverse primer	0.5µl
Sample DNA	2.0µl
Nuclease free water	9.5µl
Total volume	25 µl

- **PCR reaction mixture for *fli*C gene**

Taq buffer (5X)	2.5µl
$MgCl_2$ (25mM)	1.5µl
dNTP mix (10mM)	2.0µl
Forward primer	0.5µl
Reverse primer	0.5µl
Sample DNA	2.0µl

Taq DNA Polymerase (5u/µl)	1.0µl
Sterile water	15.0µl
Total volume	25 µl

3.8.3: Detection of PCR amplicons in agarose gel

The PCR amplicons of the bacterial 16s rRNA genes and *fli*C genes were resolved on 1% agarose gel containing ethidium bromide following methods described below (section 3.8.4). 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 (100bp/500bp ladder, Promega) run parallel to samples.

3.8.4: Gel electrophoresis

Purified genomic DNA was subjected to agarose gel electrophoresis performed in a submarine gel electrophoresis system (Bangalore genei (India) Pvt. Ltd., India). Agarose was suspended in 1X TAE buffer (1%) and dissolved by heating in a water bath until clear solution was obtained. The solution was allowed to cool down to about 50-60^{o}C. Ethidium bromide (0.5µg/ml) was added mixed and poured into the gel casting tray. The solution was allowed to solidify and then it was completely submersed in electrophoresis tank containing 1X TAE running buffer. DNA sample (6µl) and gel loading buffer (2µl) was mixed and loaded onto wells. Electrophoresis was run at 60volt for 1 hour. The gel was then picked up from the tank and viewed under UV light in a UV transilluminator (Bangalore Genei (India) Pvt. Ltd, Bangalor, India). Bright fluorescent orange bands indicated the presence of DNA.

3.9: Cloning and sequencing of purified PCR product:

The pGEM-T vector (Promega, Madison, USA) was used for the cloning of purified nucleotide sequence (PCR products) following the method of Sambrook and Russel (2001).

3.9.1: Competent cell Preparation:

The *Escherichia coli* strain JM109 was used for the procedure. One cfu of fresh overnight grown culture of *Escherichia coli* strain JM109 was added to 10 ml Luria-Berteni broth (LB) and incubated at 37˚C for12-14 hours. Then 100 µl broth culture (overnight grown) was inoculated into same volume of freshly prepared LB broth and incubated for 2 to 4 hours at 37˚C. Then centrifugation has done at 6000 rpm for 7 min to harvest the bacterial cells in1.5 ml microcentrifuge tube. After discarding the supernatant, 750 µl of solution- I (solution of 80mM $MgCl_2$ and 20mM $CaCl_2$) was added and mixed gently. Again centrifuged at 3000 to 4000 rpm for 5 to 7 min and supernatant was discarded. Then 750 µl solution - II (100mM $CaCl_2$ solution) was added as same volume as solution - I and mixed gently. The mixture was incubated for 45 minutes at 4˚C. After incubation the mixture was taken for centrifuge at 3000 to 4000 rpm for 5 to 7 minutes and supernatant was discard. Then finally 500 µl of solution – II was added and stored at -70°C.

3.9.2: Transformation:

A suitable quantity (2 to 10 µl) of ligation mixture and 100 µl of competent cell suspension was mixed carefully and the mixture was incubated at 4°C for 30 to 60 minutes. Then the heat shock therapy was applied by incubating the solution at 42°C for 90 seconds and again at 4°C 10 minutes. 300 µl LB was added to the solution and incubated at 37˚C for 2 hours in shaker incubator (Remi, India). The centrifugation has done at 6000 rpm for 5 minutes for harvesting the transformed bacterial cells and supernatant was discarded. Finally the precipitate was resuspended with 100 µl of fresh LB broth and plated in 20 ml LB agar plate containing 4 µl 20% Isopropyl thiogalactoside, or isopropyl beta-D-thiogalactopyranoside (IPTG), 20 µl 4% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and 20 µl 50 mg/ml ampicillin.

- **Ligation mixture**

Ligation buffer:	5.0 µl
Ligation buffer:	5.0 µl
pGEM-T vector:	0.5 µl
T4 DNA ligase:	1.0 µl
Purified RT-PCR product:	1.5 µl
Sterile distilled water:	2.0 µl

- **Blue-white Screening**

Among the blue and white colonies in the ampicillin plate a single white colony was isolated and picked up through a sterile tooth pick and kept into a 1.5 ml micro centrifuge tube containing 200 µl of sterile distilled water. Then the mixture was taken to boil at 100°C for 10 minutes and cooled immediately, then centrifuged at 7000 rpm for 5 minutes at 4˚C. Finally the supernatant was transferred into a fresh 1.5 ml micro centrifuge tube. For PCR reaction 2 µl supernatant was used.

- **PCR reaction mixture for cloned DNA**

Taq buffer (5X)	5µl
$MgCl_2$ (25mM)	1.5µl
dNTP mix (10mM)	1µl
Forward primer	1 µl
Reverse primer	1 µl
Sample DNA	2.0µl
Taq DNA Polymerase (5u/µl)	0.125µl
Sterile water	13.375µl
Total volume	25 µl

3.9.3: Purification and sequencing of PCR products

GeneiPure™ Quick PCR purification kit was used for purification of the PCR products. Purification was done following the manufacturer's protocol. Binding

Buffer (5 volume) was added to PCR product (1 volume) and mixed well. GeneiPure™ column was placed into a 2 ml collection tube and the sample was loaded and centrifuged at 11,000 rpm for 1 minute. The flow through was discarded and the GeneiPure™ column was again placed in the collection tube and washed with of Wash Buffer I (500 µl) and centrifuged (at 11,000 rpm for 1 minute). Flow through was discarded and the GeneiPure™ column was again kept in to the collection tube. After that, one volume of Wash Buffer II was diluted with four volume of absolute ethanol (just before use). Again, the GeneiPure™ column was washed with 700 µl of diluted Wash Buffer II and centrifuged at 11,000 rpm for 1 minute. Flow through was discarded and the said column was placed back to the collection tube. It was then centrifuged at 11,000 rpm for 2 minutes to remove the traces of Wash Buffer and the collection tube was discarded. Thereafter the column was opened and placed in fresh and sterile 1.5 ml vial. It was then incubated at 70° C for 2 minutes for complete removal of ethanol. Finally, For elution of DNA, 50 µl of pre-warmed (in dry bath at 70° C for 5 minutes) Elution Buffer was added to the center of the column and incubated at room temperature for 1-2 minutes.

3.9.4: Sequencing of the cloned insert:

The cloned PCR products were sent to Xcelris Genomics Ltd. for sequencing. Annotation and BLAST analysis were done and deduced amino acid sequences were submitted to GenBank and necessary accession numbers were procured.

3.10: Phylogenetic analysis:

The 16s rRNA gene sequences and the *fli*C gene sequences of the pathogenic fungal strains were compared with other related gene sequences available in GenBank databases using the BLAST search facility at NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul et al. 1997). Thereafter the sequences were aligned with sequences of GenBank. The nucleotide identity matrices were formed with SDT 1.2 (Muhire et al. 2014). Neighbour-joining method of Saitou & Nei (1997) was used through Kimura-2 parameter in MEGA 6.0 (Tamura et

al. 2013) to generate phylogenetic trees 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).

3.11: Bacterial wilt disease management by indigenous isolated bacteria:

3.11.1: Screening and Isolation of antagonistic bacteria:

Procedure of isolation and screening of antagonistic bacteria from fields of the present study area have been presented in section 3.4.2.

3.11.2: Biochemical characterization of bacterial antagonists:

After Gram staining all such bacteria isolated were observed under light microscope to confirm their Gram positive nature. Finally the major biochemical tests were carried out for the identification of the isolates such as Catalase test, Citrate test, Indole test, Oxidase test and Voges Proskauer test as discussed in section 3.6.

3.11.3: Molecular characterization of bacterial antagonist:

The procedure of molecular characterization of bacterial antagonist by 16s rRNA study was same as discussed in case of bacterial pathogen R. solanacearum and has already been discussed in sections 3.8 3.9 & 3.10.

3.11.3: *In-vitro* antagonism study:

For the *in-vitro* study dual culture technique was followed. In nutrient agar plate one line of pathogenic bacteria was streaked by a sterilized needle, so that, the line is passed through the centre of the Petriplate. Thereafter one lines of antagonistic bacteria was streaked parallel to that line and left side and another line on the right side of the central line. Then the plates were

incubated at 30°C. Average breadth of the lines was observed and recorded for antagonism, if any, after 12 h, 24 h and 48 h.

3.11.4: *In-vivo* antagonism study:

3.11.4.1: Root disease assessment following antagonist application (By soil application)

At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. The pot was treated as inoculated control. In second set another 1.5 Kg sterilized soil was mixed with 100 ml of *Ralstonia solanacearum* (RSG01) suspension and also with 100ml of antagonist (*Bacillus cereus*; isolate HS01). Thereafter the soil was poured in a pot. Similarly third and fourth sets were prepared with broth of another two antagonistic isolates (HS02 and HS03). Fifth set was done where a mixture of three antagonistic isolates in equal volume was added. These sets were considered as inoculated-antagonist applied sets. All these soils were poured in separate pots and labeled. Sixth set was un-inoculated control where no application of antagonistic bacteria was done. Actually this set contained sterilized soil in a pot.

Healthy tomato plants of 25 days old were sown in the pots. For each treatment four plants were sown. Reduction of disease was calculated for each plant and finally average of three plants was recorded on the basis of wilting index. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.11.4.2 Challenge inoculation by pathogenic bacteria followed by Bacterization of roots of tomato plants and assessment of disease in field condition

Bacterization of the tomato roots of 25 days old plants (of cv.PKM1) was done by dipping in bacterial suspensions for two hours. After incubation, the plants were sown in soils in plots prepared in the experimental garden of the Department. After 24 hours, the plants were inoculated with pathogenic bacteria suspension. Bacterial suspensions were made from 48 hour old cultures in Nutrient broth. The 50 ml culture was centrifuged and the pellet of bacterium was suspended in 50 ml sterile distilled water. The resultant suspension was used for inoculation. About 50 ml suspension of pathogenic bacterium was applied at the base of each plant sown in the experimental plots except the plants of control plots. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.12: *In vitro* control of *Ralstonia solanacearum*

3.12.1: *In vitro* control of *Ralstonia solanacearum* with *Trichoderma harzianum* (following dual culture technique):

At the onset of the study one fungus *Trichoderma harzianum* (IRCC-4572) was cultured in potato dextrose agar (PDA) medium contained plate. Fresh sterilized PDA plates were taken for dual culture test. Mycelia block of 6 mm diameter were cut from a 5-day old culture plate of *T. harzianum* and was placed 2 cm away from the centre of the plate. One bacterium (*R. solanacearum* isolate RSG01) was streaked in straight line in the opposite side of the mycelia block and 2 mm apart from the centre of the same plate. Growth of bacterium and fungus were recorded.

3.12.2: *In vivo* evaluation of *Trichoderma harzianum* for control of disease*:*

In the present study one *Trichoderma harzianum* (IRCC-4572) was used against the wilt causing bacteria *Ralstonia solanacearum* isolate of tomato under pot condition following the method of Kumar and Ganesan (2006) with some modifications. At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. In a similar pot 100ml of *Trichoderma harzianum* spore suspension was mixed after inoculation with *Ralstonia solanacearum* (RSG01) broth. For this study 25 days old seedlings of cv. PKM-1 were taken. The fungal spore suspension was prepared from PDA plate grown at 25°C for 96 hours. The spores from the surface were suspended in sterile distilled water and were shaken well to make a homogenized spore suspension. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.13: Control of pathogenic bacteria by botanicals:

As chemicals are not much effective to control the bacterial wilt caused by *Ralstonia solanacearum*, some botanicals were used because of their natural origin and biodegradable nature. There are some reports of effective antibacterial activity.

- **Selection and collection of plants/plant parts for extraction:**

Eight plants were selected for extraction of botanicals. The selection was based on some previous reports and local availability. The plants used for the

purpose have been listed below along with their common name, family and parts used for extraction.

- **List of plants:**

Botanical name with family, parts used and common name	**Traditional uses**	**Anti bacterial use if any**
Spermacoce hispida L. (Family: Rubiaceae; Parts used: Twigs; Common name: Shaggy button weed)	Antifungal, anticancer and antioxidant properties. (Meti et al, 2013)	Extract used against bacteria. (Muthu et al. 2010)
Allium sativum L. (Family: Amaryllidaceae; Parts used: Bulbs; Common name: Garlic)	Stimulant, diuretic, expectorant, in flatulency and dysentery (Saha et al., 2005)	Extract used against bacteria. (Wolde et al., 2018)
Zingiber officinale Rosc. (Family: Zingiberaceae; Parts used: Rhizome; Common name: Ada)	Used in dyspepsia and flatulent colic (Saha et al., 2005)	Extract used against bacteria. (Malu et al., 2009)
Azadirachita indica A. Juss (Family: Meliaceae; Parts used: Leaves; Common name: Neem)	Applied to boils as poultice, decoction antiseptic, used in ulcers and eczema (Saha et al., 2005)	Extract used against *Ralsonia solanacearum* (Narasimha and Srinivas, 2012)
Ocimum sanctum L. (Family: Lamiaceae; Parts used: Leaves; Common name: Tulsi)	Fresh leaves bruised and applied externally for curing ring worm and other skin diseases	Antibacterial (Singh et al., 2005; Mishra and Mishra 2011)

	(Saha et al., 2005)	
Cinamomum tamala (Buch.-Ham.) T.Nees & C.H.Eberm (Family: Lauraceae; Parts used: Leaves; Common name: Tejpata)	Medicinal use in skin rashes, earaches, and rheumatism also used in cooking. (Batool et al.,2020)	antibacterial, antiviral, antifungal activity, insect repellant activity, (Batool et al.,2020)
Camellia sinensis (Family: Theaceae; Parts used: Leaves; Common name: Tea)	Traditionally used as beverage drink. Several health benefits are there in tea	Antibacterial (Kawamura and Takeo,1989; Vasudeo and Sonika, 2009)
Thuja occidentalis (Family: Cupressaceae; Parts used: Leaves; Common name: Eastern white ceder)	Traditionally used in bronchitis, Rheumatism (Tsiri et al., 2009)	Antibacterial (Sah et al., 2017)

- **Alcoholic extraction Protocol:**

Plant parts (100g) were taken and cut into small pieces. The sample was mixed with 50 ml of sterilized distilled water and macerated in a pestle and morter under aseptic condition. After maceration 50ml of absolute alcohol was added. The mixture was kept for one hour in a sealed glass tube. After one hour the mixture was squeezed through a sterilized muslin cloth so as to get a crude liquid extract. The crude extract was centrifuged at 1000 rpm for 5 minutes. The filtrate was concentrated so that alcohol is removed. Again 50 ml sterile distilled water was added to it and final volume was adjusted to 100 ml. Thus concentration of crude extract became 1g/ml.

- ***In-vitro* evaluation of plant extracts:**

Extracts of the selected plants were screened at different dilutions viz., Crude extract, 50%, 25% and 12.5% diluted. The efficacy of the extracts was tested by the zone of inhibition assay technique against *Ralstonia solanacearum* causing bacterial wilt of tomato. Bacterial suspension (5 x 10^8cfu/ml) was seeded to the sterilized nutrient agar medium by mixing the bacterial culture with the cooled nutrient agar (40 to 50°C) in a 500ml conical flask. The seeded medium was poured in sterilized Petri plates and was allowed to solidify. Five wells each of 5 mm diameter were dug in each plate. One well at the centre and four wells were dug at peripheral region keeping some distance from the margin. In the central well 50 µl of sterile distilled water was placed. In peripheral wells 50 µl plant extracts each of four different concentrations were placed. The plates were incubated first at 5±1°C for 5 hours to allow the diffusion of the extract into the medium. The plates were then incubated at 28±1°C for 72 hours. Diameter of zone of inhibition produced around the wells in each plant extract at different dilutions were measured and recorded. No inhibition zone was observed around central well kept as control.

- ***In-vitro* evaluation of plant extracts:**

In the present study one plant extract (leaf extract of Camellia sinensis) was used against the wilt causing bacteria *Ralstonia solanacearum* isolate of tomato under pot condition following the method of Kumar and Ganesan (2006) with some modifications. At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. In a similar pot 100ml of *Camellia sinensis* leaf extract was mixed after inoculation with *Ralstonia solanacearum* (RSG01) broth. For this study 25 days old seedlings of cv. PKM-1 were taken. The fungal spore suspension was prepared from PDA plate grown at 25°C for 96 hours. The spores from the surface were suspended in sterile

distilled water and were shaken well to make a homogenized spore suspension. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.14: Induction of Disease resistance

3.14.1: Application of inducer-chemicals on tomato plants and inoculation

Roots of 15 days old fresh healthy tomato plants of (cv. PKM-1) were soaked in inducer chemicals [dissolved in sterile distilled water (10^{-3} M)] for 1 hour and finally sown in fresh earthen pots containing sterilized soil. Tree different chemicals *viz.* β-aminobutyric acid (BABA), Salicylic acid (SA) and Abscisic acid (ABA) were used as inducers to elicit resistance in the plants. The inducers were mixed with Tween-20 before spraying in order to ensure adhering.

One hour after treatment, treated plants were challenge inoculated with bacterial suspension of *R. solanacearum*. Experiments were performed in four sets (untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated). Plants were maintained in green house in a sterile environment under normal daylight condition.

The roots of untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated plants were harvested for studying expression of defense related enzymes after 0 day to 5 day at one day intervals following inoculation by the pathogen. Disease index was also recorded and computed and compared assessment of induction if any.

3.14.2: Extraction and estimation of defense related enzymes

Three different defense related enzymes were studied because of their potentiality to elicit defense against bacterial pathogens. The three different enzymes were Phenylalanine ammonia lyase, Peroxidase and polyphenol oxidase.

3.14.2.1: Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5):

- **Extraction:**

PAL activity was determined following the method of Sadasivam and Manickam (1996). PAL activity is the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290nm. One gram of root was harvested washed and dipped in liquid nitrogen for 10 minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 0.25M borate buffer (pH-8.7). The paste was filtered through muslin cloth. The filtrate was centrifuged [at 12500g for 15 minutes] at 4 °C. The supernatant was used as enzyme stock.

- **Enzyme assay:**

Borate buffer [0.25M] (0.5ml), Enzyme stock (0.25ml), Distilled water (1.5ml) and L-phenylalanine [0.1M] 1ml were mixed. The mixture was kept at 30°C for 30 minutes for reactions. Reaction was stopped by 0.5ml of 1M Trichloroacetic acid. Absorbance was recorded at 290nm in UV-VIS spectrophotometer. Enzyme activity was represented as μmol $min^{-1}g^{-1}$ fresh weight tissue using trans cinnamic acid as standard. PAL activity =

(Concentration x Dilution factor x Final volume x 3) / (Initial weight x Volume of enzyme x 60)

3.14.2.1: Peroxidase (POD) (EC 1.11.1.7):

 # Extraction:

POD activity was tested following the method of Hammerschmidt *et al.,* 1982. One gram of root was harvested washed and dipped in liquid nitrogen for 10

minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 0.01M sodium phosphate buffer (pH 6.5). The paste was filtered through muslin cloth. The filtrate was centrifuged [at 6000g for 15 minutes] at 4 °C. The supernatant was used as enzyme stock.

 # Enzyme assay:

Guaiacol [0.05M] (1.5ml) and Enzyme stock (200µl) were mixed in a cuvette which was placed in a UV-VIS spectrophotometer and initial reading was adjusted to zero at 420 nm. H_2O_2 (1% v/v) 100 µl was added in the cuvette and the changes in absorbance values was recorded up to 5 minutes at 1 minute intervals. Change in absorbance [$\Delta A_{420} min^{-1} g^{-1}$ fresh weight tissue] of 0.001 was considered as unit of enzyme activity. Peroxidase activity =

(Mean OD x Final volume x 10 x Dilution factor) / Initial weight

3.14.2.2: Poly-phenol oxidase (PPO) (EC 1.14.18.1):

 # Extraction

PPO assay was performed following the method of Sadasivam and Manickam (1996). One gram of root was harvested washed and dipped in liquid nitrogen for 10 minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 50mM Tris-HCl buffer (pH-7.2) containing 0.4M sorbitol and 1.0mM NaCl. The paste was filtered through muslin cloth. The filtrate was centrifuged [at 12000g for 10 minutes] at 4 °C. The supernatant was used as enzyme stock.

 # Enzyme assay:

Sodium phosphate buffer [0.1M, pH-6.5] (2.5ml) and Enzyme stock (0.2ml) were mixed in a cuvette which was placed in a UV-VIS spectrophotometer and initial reading was adjusted to zero at 495 nm. Catechol [0.01M] 0.3ml was added in the cuvette and the changes in absorbance values were recorded up to 5 minutes at 1 minute intervals. Enzyme activity was expressed as change in

absorbance. Enzyme activity = K*($\Delta A min^{-1}$) µmol $min^{-1} g^{-1}$ fresh weight tissue. PPO activity =

K x ΔO.D. x Final volume x dilution factor x 10 / initial weight x 1 minute.

[Where K=0.272 for PPO]