CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS:

3.1.1. Infected tomato fruit for pathogen isolation:

Pseudomonas syringae pv. *tomato* infected tomato fruits were procured locally from fields of Noida and IARI, New Delhi.

3.1.2. Host plants:

Tomato seeds of local and F1 (Roopsi variety, Century seeds) varieties were obtained from seed dealers in Delhi.

3.1.3. Neem fruits:

Mature neem fruits with green fruit coat but hard kernel were obtained from a single neem tree growing in the Amity University campus.

3.2. METHODS:

3.2.1. Raising of host plants:

Surface-sterilised tomato seeds (both local and F1 varieties) were sown in sterilized soilrite in plastic trays [35cm (L) × 25cm (W) ×6 cm (H)]. The plants were raised in a sterile culture room maintained at 25±1ºC with a relative humidity (RH) of 70% and 12 h of light and dark photoperiod. Trays were watered daily with sterile distilled water and once a week with sterilized Hoagland's solution as nutrient supplement.

Figure 3.1. Raising of host plants in sterile culture room under controlled conditions.

3.2.2. Aqueous Neem fruit extract preparation:

About 20g of fully mature but green neem fruits (fruit coat green but seed hard) were washed twice with sterilized Type I water and dried under aseptic condition. Seeds were macerated in 100 ml of sterilized Type I water in a pre-chilled pestle and mortar. The extract thus obtained was filtered through four folds of Muslin cloth and the filtrate was centrifuged at 8000xg at 4°C for 30 min. The supernatant obtained was filtered through 0.45 μm membrane filter and used as aqueous neem fruit extract.

3.2.3. Isolation of *P. syringae* pv. *tomato* from infected tomato fruit:

P. syringae pv. *tomato* was isolated from naturally infected tomato fruits. The infected fruit was surface sterilized using 70% ethanol for 5 sec, followed by 30 sec exposure of 0.6% Sodium hypochlorite solution, after which they were given two 30-sec rinses in sterile Type I water. The infected lesions in fruit were scrapped using a sterile scalpel and homogenized in sterile Type I water. Dilutions were plated onto *Pseudomonas* specific King's B agar containing 0.1mg ml^{-1} of cycloheximide. After incubation at 25±1℃ for 24h, single colony was isolated and used for further analysis.

3.2.3.1. Characterization of *Pst* by morphological, biochemical and physiological tests:

The single isolated colony obtained from above mentioned procedures was used further for characterization and identification of the pathogen by morphological, biochemical and physiological tests. All the tests were performed in three replicates each. *Pst* was identified by employing the following tests described earlier [Lelliott and Stead (1987); Goszczynska *et al*. (2000); Schaad *et al*. (2001); Karimi-Kurdistani and Harighi (2008) and Milijašević *et al*. (2009)]: Gram's staining; Indole production test, Methyl red test, Voges–Proskauer test, Citrate test (IMVIC tests); fluorescence on King's B agar medium; motility test; growth at 4°C and 41°C; oxidative-fermentative metabolism of glucose (O/F test); starch hydrolysis; tween 80 hydrolysis; gelatin liquefaction; aesculin hydrolysis; catalase activity; NH_3 production (Urease test); acid production from sorbitol, mannitol, inositol, erythritol and L-lactate; nitrate reduction test; ice nucleation test; Levan formation, Oxidase activity, Potato rot test, Arginine-dehydrolase activity, Tobacco hypersensitivity (LOPAT tests); KOH solubility test.

3.2.3.1.1. Gram's staining:

A colony of the bacteria was placed on a drop of water on a pre-sterilised slide and heat-fixed by carefully passing the slide over the flame three times.

1. A drop of crystal violet was added to the sample and incubated for 1 minute. The slide was rinsed with a gentle stream of water for a maximum of 5 seconds to remove unbound crystal violet.
2. A drop of Gram's iodine was added to the sample and incubated for 1 minute.
3. The slide was rinsed with 75 % alcohol for about 3 seconds and then with a gentle stream of water.
4. A drop of safranin was added to the slide and incubated for 1 minute. Subsequently it was washed with a gentle stream of water for a maximum of 5 seconds.
5. The slide was examined under the microscope.

3.2.3.1.2. IMVIC tests:

3.2.3.1.2.1. Indole test:

1. Tryptone broth tubes were drop-inoculated with the bacteria and incubated at 37°C for 2 to 5 days.
2. 1 ml Kovac's reagent was added to the tube, gently shaken and observed for the color at the interface of the broth and the reagent.

3.2.3.1.2.2. Methyl-red test and Voges-Proskauer test:

1. The tubes containing MR-VP broth were drop inoculated with the bacteria and incubated for 2 days at 37˚C.
2. 5 drops of methyl red were added to one tube and color change was observed.
3. 0.6 ml of alpha-naphthol was added to another tube and shaken gently.
4. 0.2 ml of 40% KOH was added and color change was observed.

3.2.3.1.2.3. Citrate Utilization Test:

1. Slants of Simmon's citrate agar were inoculated with a single colony of the bacteria and incubated overnight at 37˚C.
2. The color change was observed.

3.2.3.1.3. Fluorescence test:

1. King's B agar plates were streak-inoculated with the bacteria and incubated overnight at 37˚C.
2. The plate was placed on a UV-visible transilluminator and observed for the fluorescence.

3.2.3.1.4. Motility test:

1. Nutrient agar slants were inoculated with actively growing bacteria with a sterile needle upto about 1 cm deep into the slant.
2. The slants were incubated at 37˚C for 24 to 48 h.
3. The growth pattern of the bacteria on the slant (whether growing away from the axis of inoculation or not) was observed.

3.2.3.1.5. Growth at 4˚C and 41˚C:

1. King's B agar plates were streak-inoculated with the bacteria and incubated at 4˚C and 41˚C respectively for 24 to 48 h.
2. The plates were observed for bacterial growth.

3.2.3.1.6. Oxidative fermentation of glucose test:

1. Tubes of Hugh and Leifson's OF basal medium were stab-inoculated upto 3/4th of the media.
2. One of the two tubes was overlayed with 1 cm of mineral oil.
3. The tubes were incubated at 35˚C for 48 h.
4. The color change was observed.

3.2.3.1.7. Starch hydrolysis test:

1. Nutrient agar plates containing 0.2 % starch were streak-inoculated with the bacteria and incubated at 37˚C for 2 to 7 days.
2. The plates were flooded with iodine solution.
3. The plates were observed for a clear zone around the bacterial growth.

3.2.3.1.8. Tween 80 hydrolysis:

1. Nutrient agar plates containing 0.01 % $CaCl_2$ and 1 % tween 80 were streak-inoculated with the bacteria and incubated at 37˚C for upto 7 days.

2. The plates were observed for the appearance of an opaque zone around the growth.

3.2.3.1.9. Gelatin liquefaction test:

1. Nutrient agar tubes containing 12 % gelatin were stab-inoculated with the bacteria and incubated at 20˚C for 20 days.
2. The tubes were incubated at 4˚C for 15 minutes and then observed for the liquefaction of gelatin.

3.2.3.1.10. Aesculin hydrolysis test:

1. Slants of aesculin containing nutrient media were streak-inoculated with the bacteria and incubated at 37˚C for 2 to 5 days.
2. Change in the color was observed.

3.2.3.1.11. Catalase activity test:

1. A single growing bacterial colony was placed on a sterile slide.
2. A few drops of 30 % H_2O_2 were added to the colony.
3. The slide was observed for the formation of gas bubbles.

3.2.3.1.12. Urease test:

1. Nutrient broth tubes containing 0.02 % Urea were prepared.
2. The tubes were drop-inoculated with bacterial suspension and incubated at 37˚C for 7 days.
3. Change in the color was observed.

3.2.3.1.13. Acid production from sorbitol, mannitol, inositol, erythritol and L-lactate test:

1. Mineral media broth (0.1 % $NH_4H_2PO_4$, 0.02 % KCl, 0.02 % $MgSO_4.7H_2O$ and 0.008 % bromothymol blue) tubes containing 0.3 % carbohydrate (either of sorbitol, mannitol, inositol, erythritol or L-lactate) were prepared.
2. The tubes were inoculated with a growing culture of the bacteria and incubated at 25°C for 15 days.
3. The tubes were observed for the color change.

3.2.3.1.14. Nitrate reduction test:

1. Nutrient broth tubes containing 1 % potassium nitrate inoculated with the bacteria and incubate at 35°C to 37°C for 24 to 48 h.
2. To the tubes, 5 drops each of 0.8 % Sulfanilic acid solution in 5N acetic acid and 0.6 % N,N-Dimethyl-1-naphthylamine solution in 5N acetic acid were added.
3. Change in the color was observed.

3.2.3.1.15. Ice nucleation test:

1. An opaque suspension of the bacterial isolate (about 10^8 cfu ml^{-1}) was prepared.
2. Ten drops (10 µl each) of cell suspension were pipetted onto a paraffin coated sheet of aluminum foil floating on a 70% ethanol solution maintained at –5°C.
3. The number of drops that froze within 10 min was recorded.

3.2.3.1.16. LOPAT tests:

3.2.3.1.16.1. Levan test:

1. Nutrient agar plates containing 2% sucrose were prepared. The pH of the media was adjusted to 7.2 to 7.4.
2. The plates were streak-inoculated with a growing colony of the bacteria and incubated at 37˚C for 2 to 4 days.
3. The plates were observed for the appearance of dome shaped colonies.

3.2.3.1.16.2. Oxidase test:

1. Whattman's type I filter paper was saturated with 1 % tetra methyl-p-phenylenediamine dihydrochloride solution.
2. A loopful of a growing bacterial culture was rubbed onto the filter paper.
3. Change in color was observed.

3.2.3.1.16.3. Potato-rot test:

1. A potato was peeled, washed with 70 % ethanol and flamed for sterilization.
2. 7-8 mm thick slices were cut from it.
3. Each potato slice was placed in a sterile petri dish and auctoclaved water was filled upto a depth of 3-4 mm.
4. At the centre of the potato slice, a nick was made using a sterile scalpel.

5. The nick was spot-inoculated with a loopful of growing bacterial culture and incubated at 37˚C for 24 h.
6. The potato was observed for decay beyond the point of inoculation.

3.2.3.1.16.4. Arginine-dehydrolase activity test:

1. Nutrient agar tubes containing 0.001 % phenol red and 1 % DL-arginine HCl were prepared. The pH of the media was adjusted to 7.2.
2. The tubes were stab-inoculated with the bacteria.
3. The tubes were covered with a few ml of sterile mineral oil and incubated at 27˚C for 4 days.
4. Change in color was observed.

3.2.3.1.16.5. Tobacco hypersensitivity test:

1. An opaque suspension of the bacterial isolate (about 10^8 cfu ml^{-1}) was prepared.
2. A mature tobacco leaf was suface sterilized with 70 % ethanol and infilterated with the opaque bacterial suspension by forcing the suspension into the leaf with a sterile syringe.
3. The infilterated leaf was incubated at 27˚C for 24 h.
4. The leaf was observed for becoming dry and necrotic.

3.2.3.1.17. KOH solubility test:

1. A drop of 3 % KOH was placed on a clean slide.
2. A bacterial colony was picked with a sterile loop and mixed to the 3 % KOH drop on the slide.
3. The loop was lifted from the slide and observed for the formation of a mucoid thread.

3.2.3.2. Pathogenicity test:

Pathogenicity of strains was tested by stab inoculation of young, immature tomato fruits using a sterile syringe containing bacterial suspension. Control fruits were treated in the same way with water as a negative control. The inoculated tomato fruit were placed in growth chamber at 25±1˚C and symptoms were observed for seven days. After appearance of characteristic lesions on tomato fruits, the pathogen was re-isolated and Koch's postulates were confirmed.

3.2.4. *In vitro* antibacterial test:

Aqueous neem fruit extract was analysed for *in vitro* antibacterial test using standard agar well diffusion assay [Perez *et al.,* 1990]. Bacterial strains, characterized by a higher level of virulence isolated from tomato fruits were utilised at 10^8 colony forming units (CFU) ml^{-1} concentration. Uniform distribution of bacterial suspensions (0.1 ml per Petri dish) was done using spreader on *Pseudomonas* specific King's B agar medium. Wells (5mm) were made in the agar plate with a sterile cork borer. Neem fruit extract (0.1 ml) was poured into respective wells and incubated at 25±1°C for 24 h. The antibacterial activity of aqueous neem fruit extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced. This test was carried out under aseptic laboratory conditions and repeated five times, two replicates each.

3.2.5. Selection of plants of different age groups for treatment:

Plants of four different age groups i.e. 6, 8, 10 and 12 weeks after sowing were selected for experimental treatments and subsequent evaluation of the parameters.

3.2.6. Inoculum preparation for spraying on plants:

P. syringae pv. *tomato* was inoculated in King's B medium containing 0.1 mg ml^{-1} of cycloheximide and kept for incubation at 25±1˚C. After 24 h of incubation, the concentration of the culture was obtained by measuring its absorbance at 600nm (A_{600}= 0.1, equivalent to 10^8 CFU ml^{-1}). The bacteria were harvested by centrifugation of the culture at 10,000 rpm at 4^oC for 15 min. The inoculum was prepared by adjusting the concentration to 10^8 by diluting the pellet with Type I sterile water. This inoculum was used for spraying on the tomato plants. The procedure was carried out under aseptic conditions.

3.2.7. Treatment of tomato plants: The trays were divided into six groups (each group consisting of 3 replicates; each replicate having 50 randomly selected plants) for treatment. The spraying was done on the third nodal leaf of each plant. The six groups of treatment were –

1. 1^{st} group was treated with autoclaved Type I sterile water only and designated as control.
2. 2^{nd} group was inoculated with pathogen prior to Neem extract after 24 hours.
3. 3^{rd} group was treated with Neem extract prior to pathogen inoculation after 24 hours.
4. 4^{th} group was inoculated with both pathogen and Neem extract simultaneously.
5. 5^{th} group was inoculated with pathogen only.
6. 6^{th} group was treated with Neem extract only.

All the treatments were performed under aseptic conditions. Spraying was done using pre-sterilised atomizers so as to produce fine uniform droplets. Sampling was done for both treated (third nodal leaf) and untreated (distal leaves above the third node) leaves at 0, 24, 48, 72 and 96 hours intervals and after 2 weeks of the treatment. The samples were immediately stored at -20ºC and subsequently used for analysis of various parameters under study. The above procedure was repeated for each replicate of local and F1 variety.

3.2.8. Evaluation of disease severity (DS):

Disease severity in all the groups of plants was evaluated by the method described by Bhuvaneshwari (2012). In all the treatments, the untreated distal leaves were sprayed with the pathogen two days post treatment.

The lesions of bacterial speck on leaves per plant were counted. Disease severity was evaluated visually and scored using a disease index with a range of 0 to 3 (0 signifies a healthy-looking plant; 1 signifies 2 to 5 specks together or spread over each leaf; 2 signifies 6 to 10 specks; and 3 signifies more than 10 specks). Disease severity was evaluated on the basis of disease index recorded in treated as well as control plants after two weeks using the following formula:

Disease Intensity (%) = Sum of rating (0–3 scale) × 100 / (Maximum possible score × No. of leaves observed).

3.2.9. Analysis of cytoplasmic POX, PPO, LOX and Lysozyme activity:

3.2.9.1 Extraction of cytoplasmic proteins:

300mg of frozen leaf tissue was homogenized in 1.2 ml of ice cold Sodium-phosphate buffer (0.1M, pH 9.0) containing 10 mM β-mercaptoethanol, 1mM Phenyl methyl sulfonyl fluoride (PMSF), 0.001% Triton X-100, 1mM EDTA and 10 % (w/w) Polyvinylpyrrolidone (PVP) at 4^{o}C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was used as crude enzyme extract for estimation of POX, PPO, LOX and lysozyme and in-gel-activity-staining of POX, PPO and lysozyme isoforms. Each enzyme estimation assay had five replicates from 5 different samplings.

3.2.9.2. Peroxidase activity assay:

POX activity assay was carried out by making necessary modifications in the method earlier described by Neto *et al*. (2006). The reaction mixture consisted of 0.245 ml of sodium-phosphate

buffer (1M, pH 7.0), 0.25ml of Guaiacol (0.1M), 0.05 ml Hydrogen Peroxide (H_2O_2), 0.05 ml of crude enzyme extract and 1.655 ml of Type I water. The reaction mixture was incubated at 25±1°C for 5 min and reaction was terminated by addition of 0.5 ml 10% v/v Sulphuric acid. Absorbance was recorded at 470 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. The molar extinction coefficient taken for the calculation of enzyme activity for POX was, ε= 26.6 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as mM $min^{-1}g^{-1}$ fresh weight.

3.2.9.3. Polyphenol Oxidase activity assay:

PPO activity assay was carried out by the method earlier described by Bhuvaneshwari and Paul (2012). The reaction mixture consisted of 0.5 ml of sodium phosphate buffer (1M, pH 9.0), 1.25 ml of catechol (0.2M), 0.05 ml of enzyme extract and 0.2 ml of Type I water. The reaction mixture was incubated at 25±1°C for 5 min and terminated by addition of 0.5 ml 10% v/v Sulphuric acid. Absorbance was recorded at 420 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed as units g^{-1} min^{-1} fresh weight. One unit of enzyme activity was defined as the amount of enzyme required for a change in absorbance of 0.001 per minute.

3.2.9.4. Lipoxygenase activity assay:

LOX activity assay was carried out by the modifications in the method earlier described by Fortunato *et al*. (2004). The modified reaction mixture consisted of 1.955 ml of sodium phosphate buffer (0.1M, pH 7.0), 0.025 ml of Linoleic Acid (5 mM), 0.02 ml of crude enzyme extract. The reaction mixture was incubated at 25±1°C for 2 min and terminated by addition of 0.5 ml 5% v/v Sulphuric acid. Absorbance was recorded at 234 nm using UV-VIS spectrophotometer (Shimadzu, 1650). The molar extinction coefficient for LOX used was, ε=25 $mM^{-1}cm^{-1}$. Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed in mM min^{-1} g^{-1}fresh weight.

3.2.9.5. Lysozyme activity assay:

3.2.9.5.1. Substrate Preparation:

5 mg of the lyophilized cell walls of *Micrococcus lysodeikticus* was dissolved in 1 ml of sodium acetate buffer (50 mM, pH 5.0). From this stock, 60 µl of the suspension was used to arrive at 300 µg/3ml reaction mixture.

3.2.9.5.2. Lysozyme activity Assay:

Lysozyme activity was estimated as the rate of lysis of *M. lysodeikticus* cell walls with necessary modifications in the method earlier described by Sakthivel *et al*. (2010). To 250 µg of protein, 60 µl of the substrate stock as prepared above was added and the volume was made up to 2.5 ml with sodium-acetate buffer (50 mM, pH 5.2). The reaction mixture was incubated at 37±1°C for 5 minutes and the reaction terminated by addition of 500 µl of 0.1 M Sodium hydroxide. The enzyme activity was monitored by recording the absorbance at 570 nm (Shimadzu, 1650). The enzyme activity was calculated as the amount of protein required to decrease the absorbance value by 0.01 units. Enzyme activity was expressed as units ml^{-1}.

3.2.10. Statistical analysis of the data:

The data were statistically analyzed for analysis of variance (ANOVA) using the general linear model procedure and the least squares means test of the statistical software SAS (version 9.2 developed by SAS institute Inc., Cary, NC, USA). Multiple pairwise comparison tests using least-square means were performed for post-hoc comparisons after two way with treatment and time as the two factor with replications. The corrections used for multiple comparisons were Tukey's honest significantly differences test (HSD) procedure. Data for disease incidence and severity were statistically analyzed by SPSS software for windows version 16 (SPSS Inc., Chicago, Illinois, USA) using univariate general linear model procedures and one-way ANOVA respectively followed by post-hoc comparisons using Tukey's HSD.

3.2.11. Native-Basic PAGE and in-gel-activity-staining:

The isozyme profiles of acidic POX, PPO and lysozyme located in the cytoplasm were analysed by native basic PAGE [Laemmli, 1970], without SDS. Electrolyte for electrode reservoirs was Tris-glycine (pH 8.3). Bromophenol blue (0.01%) was used as tracking dye. For each sample 75 µg proteins were loaded onto the native basic polyacrylamide gel for isoform analysis. For POX and PPO, the native gel consisted of 10% resolving gel and 4% stacking gel. For Lysozyme, the native gel consisted of 8% resolving gel and 4% stacking gel. Electrophoresis was carried out at

70mA/gel for 3 hours at 4°C. After electrophoresis, the gels were stained for iso-POX by incubating in 0.1 M Sodium-phosphate buffer (pH 7.0) containing 10 mM Guaiacol and 0.75% H_2O_2 [Neto *et al*., 2006]. Acidic PPO isoforms were visualized by the modified method of Anand *et al*. (2007), by equilibrating the gel in 0.1% p-phenylene diamine followed by addition of 50 mM catechol in 0.1M Sodium-phosphate buffer (pH 7.0). The lysozyme activity on Native Basic PAGE was analyzed by modifications in the method described by Sakthivel *et al*. (2010). The separating gel was incorporated with lyophilized cell walls of *Micrococcus lysodeikticus* (0.2% w/v). After the completion of the electrophorectic run, the gel was incubated in sodium-phosphate buffer (50 mM, pH=5.0) with 1% (v/v) Triton X-100 for 2 hours at 37°C under gentle shaking. The lytic activity of lysozyme was visualized as a clear transparent zone against the dark background.

The stained isoforms were distinguished by calculating the relative distance (Rf value) [Bhuvaneshwari and Paul, 2012] of each isozyme band from each zymogram using the following equation: Rf value = Distance migrated by the isoenzyme band from the start of the resolving gel/Distance migrated by tracking dye from the start of the resolving gel.

3.2.11.1. Lysozyme band elution and concentration estimation:

The concentration of the in-gel-activity stained lysozyme band was measured by standard protocol provided by Thermo scientific. The band in the gel was excised and macerated in 500µl elution buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0.1 mM EDTA (pH=7.5) in a pre-sterilised and chilled mortar and pestle. The macerated gel piece was then placed in a clean microcentrifuge tube. The tube was incubated on a rotary shaker at 37°C overnight. After incubation, the tube was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant was carefully pipetted into a new microcentrifuge tube. This was subsequently analysed for lysozyme concentration by Bradford's method.

3.2.12. Analysis of relative gene expression levels of the defense enzymes:

Relative *g*ene expression quantification of peroxidase *CEVI-1*, lipoxygenase *TomloxD* and polyphenol oxidase *PPOA* genes was performed by real time polymerase chain reaction (qRT-PCR) using 18S gene as endogenous control. The qRT-PCR was outsourced to Xcelris Genomics Pvt. Ltd., Ahmedabad, India. As per company policy, the protocols have not been shared with us.

3.2.12.1 Primer Sequences:

The primer sequences for the three target tomato genes were obtained from www.ncbi.nlm.nih.gov by performing BLAST of the mRNA of the corresponding gene.

Gene Name	Direction	Sequence 5'-3'	Primer Length	Amplicon length
Peroxidase gene CEVI-1	F primer	GCAACAAGCCCAAAGTACCG	**20**	**219**
	R primer	GAAACAACGCCAGGACACAC	**20**	
Polyphenol Oxidase A	F primer	AATTCCTCCCGAAAGCCAGG	**20**	**375**
	R primer	TTTGGTACCAGAGTCACCGC	**20**	
Lipoxygenase Tomlox D	F primer	GCAGATCGCTAAAGCACACG	**20**	**123**
	R primer	GCGCTTAACTGCCTATGTGC	**20**	
18S rRNA	F primer	GCAAGACCGAAACTCAAAGG	**20**	**107**
	R primer	TGTTCATATGTCAAGGGCTGG	**21**	

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