CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS:

Host plant: *Solanum lycopersicum*, varieties: Local variety (F22) and F1 (Priya-6636) seeds were procured from local vendors of Delhi, NCR.

3.2. METHODS:

3.2.1. Sowing of Seeds:

Seeds of *Solanum lycopersicum* were sown in sterilized soilrite (artificial soil) in plastic trays (35 cm x 25 cm x 6 cm; L x W x H) and maintained at 25°C ±1 in a sterile culture room; with a relative humidity (RH) of 70% and photoperiod of 12 hours L/D. Trays were watered daily with sterile distilled water and weekly supplemented with of 100% sterile Hoagland's nutrient solution.

3.2.2. Sampling:

The plants were divided into two groups for both local and F1 variety. 5 week old seedlings were transferred to the organic farm of Amity University (Noida). Each group consisted of 200 plants. The apical leaf was tagged for sampling. Sampling was divided in 15 stages (weekly intervals). Both varieties were studied from bud stage till senescence. The leaves were excised from the plants using sterile forceps and were immediately placed in sterile petri dishes and brought to the laboratory. One group of plants from each variety was considered as control.

3.2.3. Isolation of Phylloplane microbiota:

Microbes were isolated by leaf imprint method. For isolation of phylloplane bacteria, the abaxial and adaxial surfaces of the leaves were pressed on nutrient agar supplemented with 0.1mg/ml of actidione (Agrios, 2005). Phylloplane microfungi were isolated by pressing the leaves on PDA medium supplemented with 0.3mg/ml streptomycin (Agostini and Timmer, 1992). The nutrient agar plates were incubated at 35±1°C and PDA plates at 25±1°C. Bacterial and fungal colonies were enumerated per sq.cm. of the leaf after 24 hrs and five days of incubation, respectively. Pure cultures of dominant bacterial and fungal

species were maintained on nutrient agar and PDA slants respectively. The process was repeated for both abaxial and adaxial surface and for all 15 stages of leaf development, for both varieties of tomato. Five replicates were taken for each leaf surface at every developmental stage of each variety.

3.2.4. Identification of Phylloplane isolates:

The fungal species were identified microscopically by studying the morphology of the mycelium, fruiting bodies and sporulating structures after staining with Lacto-phenol cotton blue. The species were identified by referring to standard identification manuals. The bacteria were identified by 16S rRNA sequencing. InstaGene TM Matrix Genomic DNA isolation kit was used to isolate bacterial genomic DNA. Gene fragment was amplified using 16S rRNA universal primers on a MJ Research PTC-225 Peltier Thermal Cycler. 1 μL of template DNA was added to 20 μL of PCR reaction solution. 27F/1492R primers for bacteria were used for 35 amplification cycles. Amplification was performed at 94°C for 45 seconds, 55°C and 72°C for 60 seconds each. Using Montage PCR cleanup kits (Millipore), unincorporated PCR primers and dNTPs were removed from the PCR products which were sequenced using 518F/800R primers. The reactions were carried out using ABI PRISM® BigDye Terminator Cycle Sequencing Kits using AmpliTaq®. Sequence alignment was done using BLAST (NCBI) for species identification.

3.2.5. Ultrastructural studies of the phylloplane:

Leaves from aseptically and field grown plants of local variety were sampled from bud stage till senescence stage, and were scanned under scanning electron microscope (SmartSEM V05. 04. 05. 00) to study the development of trichomes and their localization patterns. Terminal leaf at each developmental stage was excised and cut into squares (1×1 cm). Samples were fixed for 5 hours in 50% (water) gluteraldehyde solution, and washed subsequently in acetone solutions of 50% for ½ hour, 70% for ½ hour, 100% for ½ hour, and 100% for 1 hour. The samples were dried and placed on the stab for scanning. Leaves were scanned on both abaxial and adaxial surfaces.

Correlation was made between the density and localization of trichomes, and the density of microbes along with their localization pattern.

3.2.6. Inoculum preparation:

The bacterial species were individually cultured in Nutrient broth supplemented with 0.1mg/ml of actidione on an orbital shaker at 25±1°C. Aliquots were drawn at regular intervals and the O.D. was determined at 600 nm. The incubation was continued till O.D. reached 0.1., where A_{600}= 0.1, equivalent to 10^8 cfu/ml [Cottenye, 2010]. The bacterial culture was then centrifuged at 10,000×g at 4°C for 15min under aseptic conditions. The pellet so obtained was resuspended in sterilized distilled water. Fungal isolates were cultured in potato dextrose broth supplemented with 0.3 mg/ml streptomycin for 5 days (120 hours) at 25±1°C and the spore count was adjusted to 3 x 10^8 spores/ml using haemocytometer.

3.2.7. Treatments of plants:

The plants were divided into 159 groups, each consisting of 5 replicates of 20 plants. The apical leaf bud was tagged which was subsequently sampled at weekly intervals. The plants were treated as follows:-

S. No.	Group No.	Metabolites/Microbe inoculated
1	1	Sterile media (control)
2	2	*Aspergillus candidus*
3	3	*A.niger*
4	4	*A.flavus*
5	5	*Fusarium oxysporum*
6	6	*Rhizoctonia solani*
7	7	*Trichoderma viride*
8	8	*Alternaria alternata*
9	9	*A.citri*
10	10	*Cladosporium cladosporioides*
11	11	*C.herbarum*
12	12	*Curvularia lunata*
13	13	*Penicillum expansum*
14	14	*A.flavus+A.alternata*
15	15	*A.candidus+A.citri*
16	16	*A.niger+C.cladosporioides*
17	17	*R.solani+C.herbarum*
18	18	*C.lunata+F.oxysporum*
19	19	*P.expansum+T.viride*
20	20	metabolites of *A. alternata*
21	21	metabolites of *A.niger*
22	22	with metabolites of *A.flavus*
23	23	metabolites of *P.expansum*

24	24	metabolites of *C.lunata*
25	25	metabolites of *C.cladosporioides*
26	26	metabolites of *C.herbarum*
27	27	metabolites of *T.viride*
28	28	metabolites of *R.solani*
29	29	metabolites of *P.koreensis*
30	30	metabolites of *S.fonticola*
31	31	metabolites of *A.alternata+A.niger*
32	32	metabolites *of A.alternata+A.flavus*
33	33	metabolites of *A.alternata.+P.expansum*
34	34	metabolites of *A.alternata+C.lunata*
35	35	metabolites of *A.alternata+C.cladosporioides*
36	36	metabolites of *A.alternata+C.herbarum*
37	37	metabolites of *A.alternata+T.viride*
38	38	metabolites of *A.alternata+R.solani*
39	39	metabolites of *A.niger+A.flavus*
40	40	metabolites of *A.niger+P.expansum*
41	41	metabolites of *A.niger+C.lunata*
42	42	metabolites of *A.niger+C.cladosporioides*
43	43	metabolites of *A.niger+C.herbarum*
44	44	metabolites of *A.niger+T.viride*
45	45	metabolites of *A.niger+R.solani*
46	46	metabolites of *A.flavus+P.expansum*
47	47	metabolites of *A.flavus+C.lunata*
48	48	metabolites of *A.flavus+C.cladosporioides*
49	49	metabolites of *A.flavus+C.herbarum*
50	50	metabolites of *A.flavus+T.viride*
51	51	metabolites of *A.flavus+R.solani*
52	52	metabolites of *P.expansum+C.lunata*
53	53	metabolites of *P.expansum+C.cladosporioides*
54	54	metabolites of *P.expansum+C.herbarum*
55	55	metabolites of *P.expansum+T.viride*
56	56	metabolites of *P.expansum+R.solani*
57	57	metabolites of *C.lunata+C.cladosporioides*
58	58	metabolites of *C.lunata+C.herbarum*
59	59	metabolites of *C.lunata+T.viride*
60	60	metabolites of *C.lunata+R.solani*
61	61	metabolites of *C.cladosporioides+C.herbarum*
62	62	metabolites of *C.cladosporioides+T.viride*
63	63	metabolites of *C.cladosporioides+R.solani*
64	64	metabolites of *C.herbarum+T.viride*
65	65	metabolites of *C.herabrum+R.solani*
66	66	metabolites of *T.viride+R.solani*
67	67	metabolites of *P.koreensis+S.fonticola*
68	68	metabolites of *P.koreensis+A.alternata*
69	69	metabolites of *P.koreensis+A.niger*
70	70	metabolites of *P.koreensis+A.flavus*
71	71	metabolites of *P.koreensis+P.expansum*

72	72	metabolites of *P.koreensis+C.lunata*
73	73	metabolites of *P.koreensis+C.cladosporioides*
74	74	metabolites of *P.koreensis+C.herbarum*
75	75	metabolites of *P.koreensis+T.viride*
76	76	metabolites of *P.koreensis+R.solani*
77	77	metabolites of *S.fonticola+A.alternata*
78	78	metabolites of *S.fonticola+A.niger*
79	79	metabolites of *S.fonticola+A.flavus*
80	80	metabolites of *S.fonticola+P.expansum*
81	81	metabolites of *S.fonticola+C.lunata*
82	82	metabolites of *S.fonticola+C.cladosporioides*
83	83	metabolites of *S.fonticola+C.herbarum*
84	84	metabolites of *S.fonticola+T.viride*
85	85	metabolites of *S.fonticola+R.solani*
86	86	metabolites of *A.alternata+A.niger+A.flavus*
87	87	metabolites of *A.alternata+A.niger+P.expansum*
88	88	metabolites of *A.alternata.+A.flavus+P.expansum*
89	89	metabolites of *A.alternata+A.niger+C.lunata*
90	90	metabolites of *A.alternata+A.flavus+C.lunata*
91	91	metabolites of *A.alternata+P.expansum+C.lunata*
92	92	metabolites of *A.alternata+A.niger+C.cladosporioides*
93	93	metabolites of *A.alternata+A.flavus+C.cladosporioides*
94	94	metabolites of *A.alternata+P.expansum+C.cladosporioides*
95	95	metabolites of *A.alternata+C.lunata+C.cladosporioides*
96	96	metabolites of *A.alternata+A.niger+C.herbarum*
97	97	metabolites of *A.alternata+A.flavus+C.herbarum*
98	98	metabolites of *A.alternata+P.expansum+C.herbarum*
99	99	metabolites of *A.alternata+C.lunata+C.herbarum*
100	100	metabolites of *A.alternata+C.cladosporioides+C.herbarum*
101	101	metabolites of *A.alternata+A.niger+T.viride*
102	102	metabolites of *A.alternata+A.flavus+T.viride*
103	103	metabolites of *A.alternata+P.expansum+T.viride*
104	104	metabolites of *A.alternata+C.lunata+T.viride*
105	105	metabolites of *A.alternata+C.cladosporioides+T.viride*
106	106	metabolites of *A.alternata+C.herbarum+T.viride*
107	107	metabolites of *A.alternata+A.niger+R.solani*
108	108	metabolites of *A.alternata+A.flavus+R.solani*
109	109	metabolites of *A.alternata+P.expansum+R.solani*
110	110	metabolites of *A.alternata+C.lunata+R.solani*
111	111	metabolites of *A.alternata+C.cladosporioides+R.solani*
112	112	metabolites of *A.alternata+C.herbarum+R.solani*
113	113	metabolites of *A.alternata+T.viride+R.solani*
114	114	metabolites of *A.alternata+A.niger+P.koreensis*
115	115	metabolites of *A.alternata+A.flavus+P.koreensis*
116	116	metabolites of *A.alternata+P.expansum+P.koreensis*
117	117	metabolites of *A.alternata+C.lunata+P.koreensis*
118	118	metabolites of *A.alternata+C.cladsporioides+P.koreensis*
119	119	metabolites of *A.alternata+C.herbarum+P.koreensis*

120	120	metabolites of *A.alternata+T.viride+P.koreensis*
121	121	metabolites of *A.alternata+R.solani+P.koreensis*
122	122	metabolites of *A.alternata+A.niger+S.fonticola*
123	123	metabolites of *A.alternata+A.flavus+S.fonticola*
124	124	metabolites of *A.alternata+P.expansum+S.fonticola*
125	125	metabolites of *A.alternata+C.lunata+S.fonticola*
126	126	metabolites of *A.alternata+C.cladosporioides+S.fonticola*
127	127	metabolites of *A.alternata+C.herbarum+S.fonticola*
128	128	metabolites of *A.alternata+T.viride+S.fonticola*
129	129	metabolites of *A.alternata+R.solani+S.fonticola*
130	130	metabolites of *A.alternata+A.niger+A.flavus+P.expansum*
131	131	metabolites of *A.alternata+A.niger+A.flavus+C.lunata*
132	132	metabolites of *A.alternata+A.niger+A.flavus+C.cladosporioides*
133	133	metabolites of *A.alternata+A.niger+A.flavus+C.herbarum*
134	134	metabolites of *A.alternata+A.niger+A.flavus+T.viride*
135	135	metabolites of *A.alternata+A.niger+A.flavus+R.solani*
136	136	metabolites of *A.niger+A.flavus+P.expansum+C.lunata*
137	137	metabolites of *A.niger+A.flavus+P.expansum+C.cladosporioides*
138	138	metabolites of *A.niger+A.flavus+P.epansum+C.herbarum*
139	139	metabolites of *A.niger+A.flavus+P.expansum+T.viride*
140	140	metabolites of *A.niger+A.flavus+P.expansum+R.solani*
141	141	metabolites of *A.flavus+P.expansum+C.lunata+C.cladosporioides*
142	142	metabolites of *A.flavus+P.expansum+C.lunata+C.herbarum*
143	143	metabolites of *A.flavus+P.expansum+C.lunata+T.viride*
144	144	metabolites of *A.flavus+P.expansum+C.lunata+R.solani*
145	145	metabolites of *P.expansum+C.lunata+C.cladosporioides+C.herbarum*
146	146	metabolites of *P.expansum+C.lunata+C.cladosporioides+R.solani*
147	147	metabolites of *C.lunata+C.cladosporioides+C.herbarum+T.viride*
148	148	metabolites of *C.lunata+C.cladosporioides+C.herbarum+R.solani*
149	149	metabolites of *C.cladosporioides+C.herbarum+T.viride+R.solani*
150	150	metabolites of *C.herbarum+T.viride+R.solani+A.alternata*
151	151	metabolites of *C.herbarum+T.viride+R.solani+A.niger*
152	152	metabolites of *C.herbarum+T.viride+R.solani+A.flavus*
153	153	metabolites of *C.herbarum+T.viride+R.solani+P.expansum*
154	154	metabolites of *C.herbarum+T.viride+R.solani+C.lunata*
155	155	metabolites of *C.herbarum+T.viride+R.solani+C.cladosporioides*
156	156	metabolites of all fungi
157	157	metabolites of all fungi+bacteria
158	158	metabolites of *P.syringae*
159	159	metabolites of all microbes+*P.syringae*

All the treatments were performed under aseptic conditions. Spraying was done using sterilised atomizers so as to produce fine uniform droplets.

Leaves were sampled at 0, 24, 48 and 72 hours of spraying. Leaves were excised with a sharp sterile blade, weighed, and processed for preparation of methanolic extracts. All the samples were collected in replicates of five.

3.2.8. Extract preparation:

Methanol extracts of the sampled microbe treated leaves were obtained as described by Fattahi *et al*. 15 grams of leaves were macerated in sterilised ice cold mortar in cold room maintained at 4°C and then extracted with 300 ml of 5% methanol by boiling for 15 min. The extract was filtered and centrifuged at 5000 rpm for 10 min. at 4°C. The supernatant was evaporated in a water bath at 80°C and stored at -20 ^{o}C for further analysis.

3.2.9. Determination of total phenolic content:

Total phenols were estimated as per the protocol described by Fattahi *et al* [2014]. 0.5 ml of the methanolic extract was mixed with 0.5 ml of 2.0 N Folin-Ciocalteu reagent. The solution was kept at 25^{o}C for 5-8 min before adding 2 ml of sodium carbonate solution (7.5 %) and subsequently adjusting the volume to 8 ml with water. After 2 hours, the absorbance was measured at 725 nm. Gallic acid was used as a standard and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: $y = 0.006x + 0.038$, $R2=0.999$, Where y is absorbance at 760 nm and x is total phenolic content in the extracts expressed in mg/gm of fresh weight.

Total phenolic content was expressed as mg gallic acid equivalents per gram of fresh weight (mg/gm).

3.2.10. Determination of total flavonoids content:

Total flavonoids were estimated as per the protocol described by Fattahi *et al* [2014]. One hundred micro litres of methanolic extract was added to 4 ml of distilled water followed by 0.3 ml of sodium nitrite (5%). After 5 min, 0.3 ml of aluminium chloride (10%) was added. Subsequently after 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted by the addition of 3.3 ml distilled water and mixed thoroughly. The absorbance was determined at 510 nm against a blank. Quercetin acid was used as standard for the calibration curve. Total flavonoids content of the extract was expressed as mg Quercetin equivalents per gram of fresh weight (mg/g).

3.2.11. Estimation of PAL and TAL:

The experiment was carried out as described by Gonzalez-Aguilar *et al*. [2004]. Frozen leaf tissue (300 mg) was homogenized in 1.2 ml of ice cold 0.05M borate buffer (pH 7.0) containing 10 mM ß-mercaptoethanol, 1mM PMSF, 0.001% Triton X-100, 1mM EDTA and 10 % (w/w) PVP at 4°C. The homogenate was centrifuged at 15000 x g for 20 min at 4^oC. The supernatant was used estimation of PAL/TAL activity. The reaction mixture for PAL/TAL consisted of 0.05 ml of enzyme extract, 0.95 ml of 0.05M borate buffer (pH 7.0), 0.11 ml of 100mM L-phenylalanine/L-tyrosine respectively. The reaction mixture was incubated in a water bath at 40°C for 30 min and reaction was terminated by adding 2% w/v trichloroacetic acid (TCA). Absorbance was recorded on UV-VIS spectrophotometer (Shimadzu, 1650) at 275 nm / 310 nm respectively for estimating PAL/TAL activity. Reaction mixture without enzyme extract served as blank. The PAL/TAL activity was calculated using respective molar extinction coefficient ε = 15.56 x 10^{-3} μM^{-1} cm-1/ ε = 9.554 x 10^{-3} μM^{-1} cm^{-1}. Enzyme activity was expressed as units mg^{-1} protein.

3.2.12. Estimation of PPO and POX:

The estimation was carried out as described by Mayer *et al* [1965]. 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 ß-mercaptoethanol, 1mM PMSF, 0.001% Triton X-100, 1mM EDTA and 10 % (w/w) PVP at 4^oC. The homogenate was centrifuged at 15000 x g for 20 min at 4°C. The supernatant was used as crude extract for estimation of activities of PPO, POX. The reaction mixture for PPO consisted of 0.5 ml of phosphate buffer (1M, pH 9.0), 1.25 ml of catechol (0.2 M), 0.05 ml of enzyme extract and 0.2 ml of distilled water. The reaction mixture for POX consisted of 0.245 ml of phosphate buffer (1M, pH 6.5), 0.25 ml of pyrogallol (0.1 M), 0.05 ml of 100 mM hydrogen peroxide, 0.01 ml of enzyme extract and 1.445 ml of distilled water. In both the cases, the reaction mixture was incubated at 25^oC for 5 min and terminated by addition of 0.5 ml of 10% (v/v) Sulphuric acid. Absorbance was recorded at 420 nm using UV-VIS spectrophotometer. Reaction mixture without enzyme extract served as blank. Enzyme activity of PPO was expressed as units mg^{-1} protein. Enzyme activity of POX was calculated using the molar extinction coefficient ε = 2.47 x 10-3 μM^{-1} cm^{-1} and expressed as units mg^{-1} protein.

3.2.13. Estimation of intercellular protein concentration:

The estimation was carried out according to the method described by Wit and Spikman [1982]. Leaves were infiltrated with sterile distilled water. For this purpose a beaker containing the leaves was filled with water (a perforated ceramic lid was placed on the surface of water to prevent the leaves from floating) and then placed in a desiccator connected to a suction pump. After 10 min under reduced pressure at room temperature, air was readmitted into the desiccator. Surface dried leaves were placed into a centrifuge tube and centrifuged for 10 mm at 3000 g. The extruded intercellular fluids (0.5 ml per leaf) were sterilized by membrane filtration (Millex-GS; 0.22 pm) and used without further dilution, or stored at - 20 °C.

3.2.14. Microbe-microbe interactions (*in vitro*):

3.2.14.1. Dual Culture Assay: The antifungal activity of the bacterial species against the isolated microfungi, was tested performing a dual culture growth assay which was done by two methods.

3.2.14.1.1. Method 1:

1ml of the diluted fungal suspension was spread plated on 30 ml Potato dextrose agar and incubated for 5 days. After the incubation period, a 1cm plug of each fungi was inoculated individually at the center of a fresh PDA plate [Sagahón *et al* 2011]. 24 hours old bacterial suspensions were spot inoculated at a distance of 2.5 cms from the inoculated fungal plug, using a sterile cotton swab. The plates were then incubated at 25±1°C and the fungal colony diameter was observed after 48 hours. The observations were repeated subsequently after every 24 hours. Control plates lacked bacterial inoculation. The percent growth inhibition (PGI) of the tested fungi was calculated using the following formula:

$$\text{PGI (\%)} = \frac{\mathbf{KR - R1}}{\mathbf{KR \times 100}}$$

where, KR denotes the distance (measured in mm) from the point of inoculation to the colony margin on the control plates, and R1 refers to the distance of fungal growth from the point of inoculation to the colony margin on the treated plates in the direction of the antagonist [Korsten and Jager 1995].

3.2.14.1.2. Method 2:

1ml of the diluted fungal suspension was spread plated on 30 ml Potato dextrose agar. On the same plate the bacterial cultures were spot inoculated at a distance of 2.5 cms from each other. The plates were incubated at 25±1°C [Jayaswal 1990 and Kerr 1999]. The diameter of zone of inhibition was measured after 48 hours, 72 hours and 96 hours. Control plates were plated with only the fungi and no bacterial inoculation was done.

3.2.15. Microbe-microbe interactions (*in vivo*):

The leaves sprayed with microbial suspensions (bacteria-bacteria, bacteria-fungus, fungus-fungus) were tested for antagonistic activity by leaf imprints at 0, 24, 48 and 72 hours of treatments. The CFU count was established to assess any inhibition. Imprints of leaves sprayed with sterile distilled water served as control.

3.2.16. Agar Well Diffusion Assay:

PDA plates were prepared and 1ml of the diluted fungal suspension was spread plated using a sterile spreader. Four 0.5 cm deep wells were subsequently created at a distance of 3cm from each other [Sawale A *et al*., 2014]. The four wells have four different bacterial metabolites to be tested against one fungus at a time.

3.2.16.1. Secondary metabolites preparation: 10 ml of bacterial suspensions (10^8 cells/ml) were centrifuged at 10000 rpm for 15 minutes at 4°C. The pellet was discarded and the supernatant was filtered through 0.22µm filter membrane. The filtrates were used as the secondary metabolites for performing the assay. 250 µl of the metabolites of each bacterial culture were added into the wells of PDA plates. The plates were incubated at 25±1°C. The zone of inhibition was recorded every 24 hours.

3.2.17. Statistical Analysis:

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 pair wise comparison tests using least-square means were performed for post-hoc comparisons after two way with treatment and time as two factor with replications. The corrections used for multiple comparisons were Tukey's honest significantly differences test (HSD) procedure.