Comparative Pathogenicity Study of *Ralstonia solanacearum* F1C1 between Eggplant and Tomato Seedlings by The Leaf Clip Inoculation Method

2.1 Abstract:

Ralstonia solanacearum, a soil-borne bacterial pathogen, is an agricultural threat infecting most of the solanaceae family crops worldwide. Its pathogenicity has been well studied in a few host plants such as tomato and *Arabidopsis thaliana*. Here, we are reporting pathogenicity of *R. solanacearum* F1C1 in eggplant (*Solanum melongena* L.), an important crop host employing two leaves stage seedlings of the host by the leaf clip inoculation method. Clipping a part of the cotyledon leaves of the two weeks old eggplant seedlings using a pair of scissors dipped in the bacterial inoculum caused aggressive bacterial wilt within one week of inoculation. Using *R. solanacearum* F1C1 strains tagged with *gus* and mCherry reporter, colonization and migration of the bacterium in eggplant seedlings was demonstrated. Furthermore, *hrpB, hrpG* and *phcA* mutant of F1C1 were created and their virulence was studied. While the *hrpB* and *hrpG* mutants were found to be non-pathogenic, the *phcA* mutant was found to be altered in its virulence in eggplant seedlings. Importantly, in a comparative study between tomato and eggplant seedlings recruited in close proximity, we could demonstrate that *R. solanacearum* was more aggressive in eggplant seedlings than in tomato seedlings. The *phcA* mutant, which is severely altered in its virulence in tomato seedlings, was found to be much more aggressive in eggplant seedlings. By counting bacterial load at different days post inoculation (DPI), we found out that bacterial load in eggplant seedlings was higher than in tomato seedlings. As the pathogenicity assay is quite simple, we believe that in future our study might be useful to find out the mechanism behind the differential aggressiveness of this pathogen in the two closely related hosts.

2.2 Introduction:

R. solanacearum causes bacterial wilt in 450 plant species of 54 botanical families including many agronomically important crops distributed worldwide [1,2]. Reporting of new hosts from different regions is quite frequent suggesting the

expansion of its host range [3,4,5,6]. Though, the pathogen is causing the same wilt symptom in different hosts, its aggressiveness in different hosts is not the same [7,8]. Therefore, under laboratory condition, only a few hosts such as tomato and *Arabidopsis* have been mainly used by scientists worldwide to understand *R. solanacearum* pathogenicity and its host adaptation. Experimental evolution research in this bacterium has revealed the role of transcription regulators in host adaptation and colonization [8].

Bacterial wilt in eggplant is a common disease in tropical and subtropical regions including India [9,10,11]. However, there is no report of the use of eggplant as a model host to study *R. solanacearum* pathogenicity at the molecular level. Most of the studies have been focused to screen resistant eggplant cultivars against the bacterial wilt disease and to understand resistant mechanisms of eggplant against the pathogen [12,13,14,15]. Eggplant being a different host, as well as from economic point of view, understanding eggplant and *R. solanacearum* interaction is of significant importance and interest.

A comparative study with regard to *R. solanacearum* adaptation and pathogenicity mechanisms in different hosts is not common in this field. There are several possible difficulties scientists might have to overcome to compare pathogenicity of this bacterium in two different host plants. First, in grown up host plants the usual soil drenching or direct stem inoculation methods do not always produce disease symptoms of similar magnitude. Secondly, it is not easy to grow and maintain different grown up hosts in close proximity during the pathogenicity so that the disease symptom can only be attributed to the pathogen infection. Therefore, there is a requirement of a stable and consistent pathogenicity assay effective in different hosts where both the hosts can be grown and maintain together under the same condition.

Earlier in our laboratory, we demonstrated that *R. solanacearum* F1C1 is virulent on the early stages of tomato seedlings by the leaf clip inoculation method [16]. In this method, the seedlings are maintained in a 1.5 or 2.0 ml microfuge tubes under gnotobiotic conditions and the pathogenicity assay is stable and consistent. Here, we have exploited this simple approach to do a comparative pathogenicity study

between eggplant and tomato seedlings by keeping the seedlings of the two different hosts within a single microfuge tube.

In this study, we have first demonstrated that leaf clip is also an efficient method to study *R. solanacearum* pathogenicity in early stages of eggplant seedlings. We have created *hrpB, hrpG* and *phcA* mutants of F1C1 which are well established virulence deficient mutants in tomato plants, to study the role of these regulatory genes in eggplant seedlings as well as to establish whether this mood of inoculation in eggplant seedlings is effective to screen virulence functions of *R. solanacearum*. We then compared *R. solanacearum* pathogenicity in tomato and eggplant seedlings; maintaining same growth conditions during the entire pathogenicity study period. We observed that *R. solanacearum* colonization as well as aggressiveness of pathogenicity was higher in eggplant seedlings in comparison to tomato seedlings of same developmental stage. So far we understand this is the first initiation of *R. solanacearum* comparative pathogenicity in two different hosts maintained in close proximity.

2.3 Material and Methods

2.3.1 Media, chemicals and lab wares

Bacterial growth media components, chemicals and antibiotics used were purchased from Hi-Media, Mumbai, India. All restriction endonucleases were procured from Fermentas (Thermo Fisher Scientific, Mumbai, India). Genomic DNA isolation kits, Plasmid DNA isolation kits, Gel-extraction kits were bought from Qiagen (New Delhi, India) and Nitrocellulose membranes (Amersham Protran, 0.45 µm NC) were bought from GE Healthcare, Germany. All Plastic wares and glasswares were purchased from Tarsons, Kolkata, India and Borosil, Kolkata, India, respectively.

Seeds of all the cultivars of eggplant and tomato were bought from Sonitpur Nursery, Tezpur, Assam, India.

2.3.2 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.1. Wild type *R. solanacearum* F1C1, derivative mutant strains and *Pseudomonas putida* were grown

in BG medium (1.0 % peptone, 0.1 % yeast extract, 0.1 % casamino acid) supplemented with 0.5 % glucose at 28°C for 48 hr [17]. 1.5 % agar was added in case of solid BG media. *Escherichia coli* were grown in 2 % LB medium [18]. 1.5 % agar was added in case of LB agar medium and was grown at 37°C for 24 hr. Concentration of antibiotics used was as ampicillin (Amp; 50µg/ml), spectinomycin (Spc; 50µg/ml) and gentamycin (Gen; 50µg/ml).

For natural transformation experiment, *R. solanacearum* F1C1 were made competent by growing them in Minimal Medium ($FeSO_4.7H_2O$, $1.25x10^{-4}$ g/l; $(NH_4)_2SO_4$, 0.5 g/l; $MgSO_4.7H_2O$, 0.05g/l; KH_2PO_4, 3.4 g/l) supplemented with 10% glycerol. The pH of the medium was adjusted to 7.0 with KOH.

Table 2.1: Bacterial strains used in this study

Ralstonia solanacearum strains			
Sl No.	**Strain**	**Relevant characteristics**	**Reference**
1	F1C1	Wild type virulent *R. solanacearum* strain (Phylotype I), isolated from wilted chili plant collected from a field nearby Tezpur University, Tezpur, India.	[19]
2	TRS1002	*rif-1 zxx::Tn5gusA11;* Gus +ve, Rif^r, Spc^r, Vir^+, derived after *Tn5gusA11* insertion in an unknown locus in the genome.	[20]
3	TRS1012	*hrpB*::Ω; Spc^r, HrpB deficient, Vir^-, Hypersensitive response deficient (HR^-), derived from F1C1	This study
4	TRS1013	*phcA*::Ω; Spc^r, PhcA deficient, exopolysaccharide deficient (EPS^-), hypermotile, derived from F1C1	This study

5	TRS1016	Gen^r, mCherry tagged F1C1	This study
6	TRS1017	*hrpB*::Ω; Spc^r, Gen^r, HrpB deficient, Vir$^-$, HR$^-$, derived from TRS1016	This study
7	TRS1018	*phcA*::Ω; Spc^r, Gen^r, PhcA deficient, EPS$^-$, hypermotile, derived from TRS1016	This study
8	TRS1027	*hrpG*::*pCZ367*; Amp^r, Gen^r, HrpG deficient, Vir$^-$, HR$^-$, derived from F1C1	This study
Other bacterial strains			
Sl No.	**Strain**	**Relevant characteristics**	**Reference**
1	MG1655	Wild type *Escherichia coli*	Lab collection
2	*Pseudomonas putida*	Tomato seedling isolate	Lab collection

2.3.3 Germination process of eggplant seeds

Bacterial wilt susceptible eggplant seeds recruited in this study was surface sterilized with 70% ethanol and then washed twice with sterile distilled water. After washing, seeds were sown on wet tissue paper and cotton bed in a plastic tray and kept for germination in a growth chamber (Orbitek, Scigenics, India) maintained at 28°C temperature, 75% relative humidity (RH) and 12 hr photo period. Every day, sterile distilled water was sprayed so that the tissue paper bed remains wet. Germination period of eggplant is little longer than tomato; seeds started germinating 5th day onwards and was allowed to grow till 13th day of transfer to growth-chamber. On 14th day, eggplant seedlings having two cotyledon leaves were transferred to sterile microfuge tubes for inoculation. Tomato (Durga; Ruby) seedlings were grown in a similar way till 6th day and 7 days old seedlings were employed for inoculation.

2.3.4 Preparation of bacterial inoculums

R. solanacearum was freshly streaked from glycerol stock stored in -80º C. For inocula preparation, freshly grown *R. solanacearum* colonies were cultured on 10 ml BG medium in a shaking incubator (Orbitek, Scigenics, India) maintained at 28°C and 200 rpm. After 24 hr incubation, bacterial cultures were pelleted down, washed with distilled water and resuspended in equal amount of sterile distilled water after centrifugation at 4000 rpm for 10 min at 4°C (5804R; Eppendorf) to make a saturated inoculums of ~10^9 CFU/ ml. Inoculums of *P. putida and E. coli* were prepared in a similar way incubated at 28°C and 37°C respectively.

2.3.5 Pathogenicity studies by the leaf clip method

2.3.5.1 Pathogenicity Assay:

Pathogenicity assay in eggplant seedlings was done by the leaf clip method as standardized previously in our laboratory for tomato seedlings [16,21]. Briefly, 14 days old eggplant seedlings were gently transferred from the germination tray to sterile 1.5 ml microfuge tubes and 1 ml of sterile distilled water was added to it. Then a pair of sterile scissors was dipped in the bacterial suspension (~10^9 CFU/ ml or other CFU as per the experimental requirement) and a portion of both the leaves from the tips were clipped off in each eggplant seedling kept in microfuge tubes. Seven days old tomato seedlings were recruited for inoculation in a similar way.

In all the experiments, 40 seedlings were inoculated in a set and each experiment was performed three times independently with two technical replicates. Seedlings mock inoculated with sterile distilled water were kept as control in all experiments. Inoculated seedlings along with control were transferred to a growth chamber (Orbitek) maintained at 28°C, 75% relative humidity and 12 hr dark and 12 hr light cycle and observed for disease progression. Up to 10^{th} day post inoculation we observed the seedlings and findings were recorded. The virulence data were analysed by Kaplan-Meier statistics [22] and log- rank test. All the steps of this clip inoculation process have been shown in Fig.2.1.

2.3.5.2 Inoculation of different eggplant cultivars:

To check the general susceptibility of eggplant seedlings to the *R. solanacearum* F1C1 by the leaf clip inoculation, we used three different eggplant cultivars namely Devkiran (Bangalore), Param Hybrid (Hyderabad) and Devgiri (Kolkata). The three cultivars of eggplant used were collected from the local vendors from the market near Tezpur University. These seeds were efficient in germination within 10-15 days. Seedlings germinated in a similar way were recruited for pathogenicity assay. Inoculation of all three cultivars was done as described above.

2.3.5.3 Inoculation of non pathogenic bacteria:

Non-pathogenic bacteria such as *P. putida* and *E. coli* were also recruited to test their susceptibility to eggplant seedlings by leaf clip inoculation in as similar way.

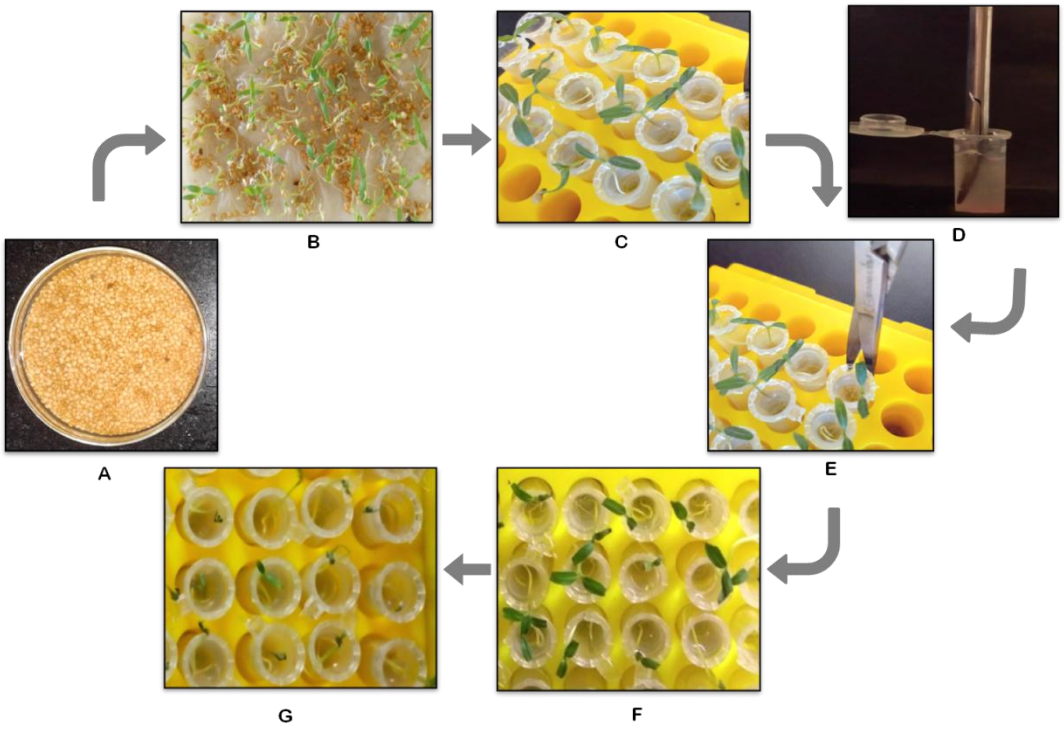


Fig.2.1: Picture depicting the different steps of the pathogenicity assay of *R. solanacearum* in eggplant seedlings by the leaf clip inoculation method. A. Surface sterilization of eggplant seeds with 70% ethanol followed by washing with sterile distilled water. B. Germination of the seeds in a wet cotton and tissue paper bed in a growth chamber maintained at 28°C and 75% relative humidity up to 14-15 days. C. Transfer of seedlings to sterile 1.5 ml microfuge tube containing water. D-E. Dipping of a pair of scissors in bacterial inoculum and clipping off a portion of both the leaves from the tips. F. Transferring of inoculated seedlings into growth chamber (28°C, 75% RH and 12 hr photoperiod) and observed for disease symptoms. G. Infected seedlings after 10 days post inoculation.

2.3.6 GUS staining of eggplant seedlings

To study the association of *R. solanacearum* with eggplant seedlings, we used *gus* marked F1C1 strain (TRS1002) to inoculate the seedlings by the leaf clip method as described above. Infected seedlings were then stained with X-gluc solution (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.1 % (v/v) Triton X-100, and 0.5 mg/ml X-gluc) following Jefferson method [23] with modification.

After 3 days of inoculation, infected eggplant seedlings were washed thoroughly with sterile water followed by $HgCl_2$ for 30 sec and 70% ethanol for 1 min. Next, seedlings were again washed with sterile distilled water. Surface sterilized seedlings were then transferred to 2.0 ml microfuge tube added with X-gluc solution and incubated for 24 hr at 37°C. After incubation, seedlings were immersed in 70% ethanol for until the clear visualization of the GUS staining.

2.3.7 Inoculation of eggplant and tomato seedlings within a single microfuge tube

To evaluate the aggressiveness of *R. solanacearum* F1C1 with respect to its two different hosts, both eggplant (15 days old) and tomato (7 days old) seedlings were transferred into a single microfuge (Fig.2.2) tube establishing same conditions for both the seedlings. The seedlings were then inoculated with F1C1 and derivative mutants such as *hrpB, hrpG* and *phcA* at concentrations ~10^9 CFU/ ml by the leaf clip method. The disease progression was recorded till 10^{th} day post inoculation.

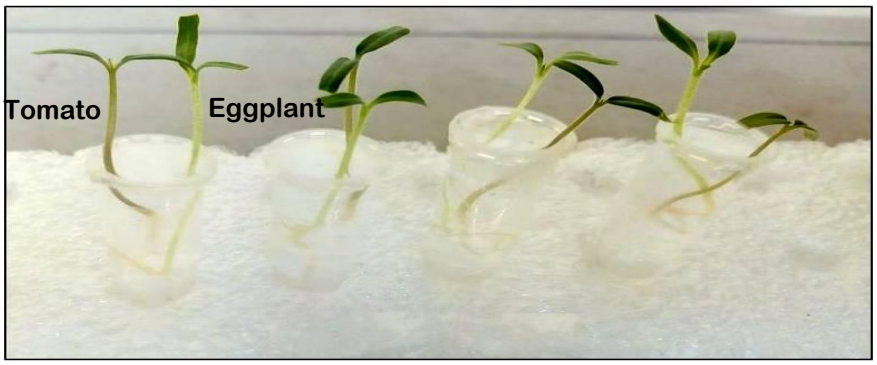


Fig.2.2: Representative picture showing pathogenicity assay set up used for comparative pathogenicity study between eggplant and tomato seedlings. Both two leaves stage eggplant and tomato seedlings were kept in a single microfuge tube and inoculated with the pathogen by the leaf clip method. Seedlings with lighter green shade of stem are eggplant seedlings.

2.3.8 Inoculation of eggplant seedlings with different concentration of *R. solanacearum* inoculums

To determine the effect of different titers of pathogen on disease progression, 15 days old eggplant seedlings were inoculated with different *R. solanacearum* F1C1 concentrations, ~10^9 CFU/ml, 10^7 CFU/ml, 10^5 CFU/ml and 10^3 CFU/ml in the inocula. Saturated inoculums of ~10^9 CFU/ml concentration was serially diluted with sterile distilled water to make 10^7, 10^5 and 10^3 CFU/ml of pathogen inoculum. Each of the inoculum was then used to inoculate eggplant seedlings by leaf clipping separately. For each dilution inoculum, 40 seedlings (Devgiri) were inoculated with two replicates and experiment was repeated three times. All the inoculated seedlings sets were incubated in a growth chamber. Seedlings were carefully observed for disease appearance and progression until the 10^{th} day post inoculation and data were recorded.

2.3.9 Transformation in *R. solanacearum* F1C1

For natural transformation of *R. solanacearum* (F1C1), methodology described by Gonzalez et al. [24] was followed with modifications in glycerol concentration used. F1C1 was grown in minimal medium added with10% glycerol as a sole carbon source for 48 hr at 28ºC in a shaking incubator set at 150 rpm. After incubation, 100 µl of the competent bacterial inoculum was gently mixed with 2-3 µg of linearized genomic DNA or plasmid DNA, spotted on 0.45µm sterile nitrocellulose membrane kept over BG-agar plate without glucose and allowed to dry-up inside the laminar air flow. After 48 hr of incubation of the plates at 28ºC, bacteria were scooped out with sterile loop and resuspended in 150 µl of distilled water. The entire bacterial suspension was then spread on the glucose added BG-agar plate containing appropriate antibiotics and incubated for 48 hr at 28ºC.

2.3.10 Creation of *hrpB and phcA* mutant of *R. solanacearum* F1C1

To create virulence regulatory mutants of *R. solanacearum,* namely *hrpB* and *phcA* in F1C1 strain background, we used genomic DNA from the *hrpB* mutant (GMI1525) [25] and *phcA* mutant (GMI1605) [26] of *R. solanacearum* GMI1000 strain which were kind gift from Stephane Genin, LIPM, France. We used genomic DNA to naturally transform wild type F1C1 individually. Further, to remove the

genomic DNA contamination of GMI1000 if any, we isolated the genomic DNA from the mutant strains of F1C1 and re-transformed wild type F1C1 separately. Next, transformants of both the genes were selected on BG-agar plates supplemented with spectinomycin antibiotic. Among many of the transformants selected, two of the transformants named TRS1012 (*hrpB* mutant of F1C1) and TRS1013 (*phcA* mutant of F1C1) were recruited in subsequent studies.

2.3.11 Creation of *hrpG* insertion mutant of *R. solanacearum* F1C1

We created *hrpG* insertion mutant by *lacZ* reporter gene fusion method (method is described in detail in Chapter 4). Taking reference sequences of GMI1000, primers were designed for partial amplification of *hrpG* homologue in F1C1 strain. Forward primer (5'-GCCAAGCTTGCGTACCGAGGCATTCAGTC-3') incorporated with *HindIII* restriction site and reverse primer (5'-GCCTCTAGATCTTGCGCAGCTTGTAGATGT-3') incorporated with *XbaI* restriction site at their 5' ends, respectively, were used to amplify approximately 500 bp amplicon of *hrpG* homolog in F1C1. Amplification was performed in 20 µl PCR reaction volume consisting of 2.0 µl of 10X Dream Taq buffer, 1.2 µl of 2 mM dNTP mix, 0.8 µl of DMSO, 0.2 µl of Dream-Taq DNA polymerase (5U/µl), 1.0 µl of 5 µM forward primer and 1.0 µl of 5 µM reverse primer. Final volume was adjusted with Nuclease-free water. PCR conditions were standardized as: (step1) initial denaturation at 95°C for 5 min; (step 2 for 35 cycles) denaturation at 94°C for 1 min; annealing at 64.5°C for 30 sec; extension at 72°C for 30 sec and (step3) final extension at 72°C for 7 min. Amplified fragment was cloned into pTZ57R/T vector and confirmed by sequencing that showed homology with *hrpG* of GMI1000. The recombinant plasmid was then sub-cloned into promoter less, insertional vector pCZ367 and resultant *hrpG*::pCZ367 construct was finally naturally transformed into F1C1.Transformant named TRS1027 was confirmed by PCR amplifications with primers oFhrpG1 (5'-GCCAAGCTTTCCAATCCATCCAGCTTCGC-3'); designed upstream of the *hrpG* cloned fragment and olacR1 (5'-AAGGGGGATGTGCTGCAAGG-3'); designed downstream of *lacZ* gene and was recruited for virulence study (Fig.2.3). We have also checked the X-gal activity of this mutant.

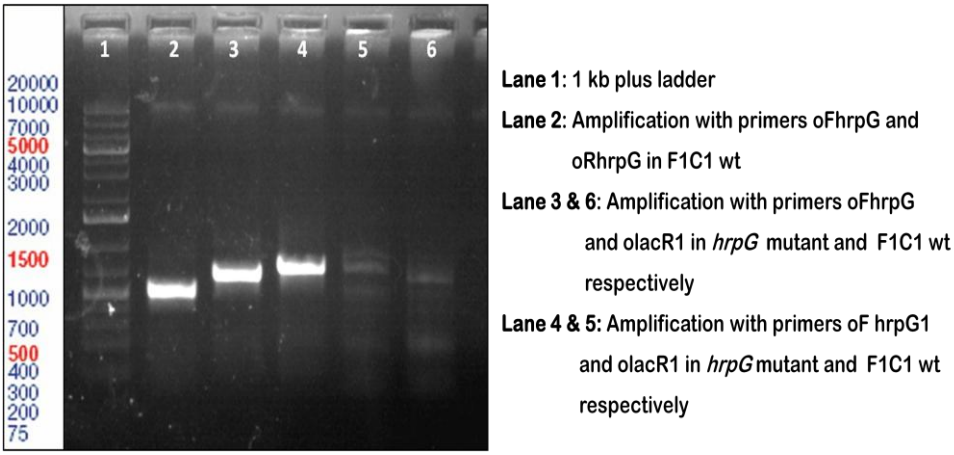


Fig.2.3: Agarose gel showing confirmation of insertion mutation in *hrpG* of F1C1. Lane1: 1 kb plus DNA ladder. Lane 2: PCR amplification of partial fragment of *hrpG* with primer pair oFhrpG and oRhrpG from F1C1 genome yielding ~500 bp size DNA band. Lane 3 and 6: PCR amplification with primer pair oFhrpG and olacR1 in *hrpG* mutant and in wild type F1C1 respectively. Amplification of ~700 bp DNA band in *hrpG* mutant, but not detected in wild type confirmed the *lacZ* insertion in *hrpG* gene. Lane 4 and 5: PCR amplification with primer pair oFhrpG1 and olacR1 in *hrpG* mutant and in wild type F1C1 respectively. Band shift showing amplification of more than ~700 bp size DNA in *hrpG* mutant confirmed the exact insertion of *hrpG* cloned fragment only in the *hrpG* gene. In F1C1 this DNA band was not apparent. The size of different DNA bands is showing at the side of the gel.

2.3.12 Confirmation of *hrpB and phcA* mutant of *R. solanacearum* F1C1

For the confirmation of mutants, we assessed *hrpB* and *hrpG* mutants of F1C1 for their hypersensitive response (HR) and *phcA* for swimming motility respectively as these two phenotypes were well established in literature. Deletion mutants of *hrpB and hrpG* mutants are reported as HR negative while *phcA* mutant is hypermotile in *R. solanacearum* GMI1000.

2.3.12.1 Hypersensitive response (HR) assay of hrpB and hrpG mutants of F1C1

hrpB (TRS1012) and *hrpG* (TRS1027) mutants were checked for hypersensitive response in tobacco (*Nicotiana tabacum*) plants. Freshly grown strains were cultured in BG medium supplemented with respective antibiotics at 28ºC, 200 rpm shaking condition for 24 hr, and suspensions were prepared as described in section 2.3.4. The bacterial suspension of concentration ~10^9 CFU/ml was used to infiltrate the tobacco leaves in the lower epidermis (ventral side) with the help of a needleless syringe. Tobacco leaves was infiltrated with F1C1 wild type as a positive control and mock inoculated with sterile distilled water in a similar way. The resulting

hypersensitive response was observed after 48 hr of infiltration. *hrpB* and *hrpG* mutants of F1C1 did not elicit any hypersensitive response in tobacco leaf unlike wild type F1C1 which confirmed the mutants (Fig.2.4).

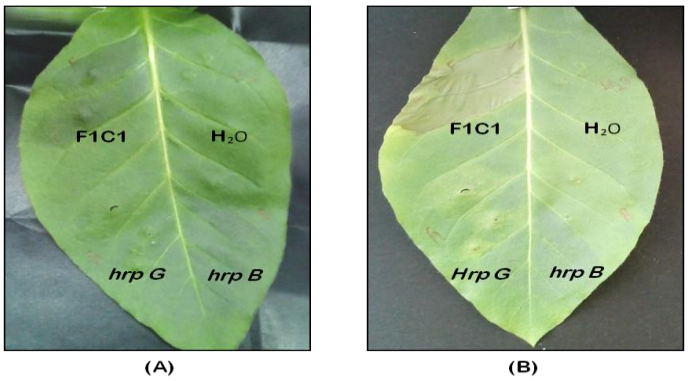


Fig.2.4: Hypersensitive response assay in tobacco leaf. Tobacco leaf was infiltrated with wild type F1C1, *hrpB, hrpG* mutants and water. F1C1 elicited hypersensitive response after 48 hr of infiltration while *hrpB and hrpG* mutants did not show any HR response. Picture has been taken (A) at 0 hr and (B) after 48 hr of infiltration.

2.3.12.2 Swimming motility assay of phcA mutant of F1C1

We assayed the *phc*A mutant for swimming motility in soft agar plates. Swim plates were prepared with BG medium added with 0.2% agar and allowed it to dry for ~ 45 min in laminar hood keeping both UV light and the blower on. TRS1013 was freshly grown on BG plates and from there strain was point inoculated with a sterile toothpick onto the prepared swimming plates. Wild type F1C1 was inoculated in a similar way from fresh colony and motility was observed after 24 hr of incubation at 28ºC.

We found *phcA* mutant generated in the background of F1C1 to be hypermotile in compared to wild type F1C1 like in case of GMI1000 [Fig.2.5 (A)].

The observation of EPS deficient colony morphology on plate further confirmed the *phcA* mutants in F1C1 [Fig.2.5 (B)].

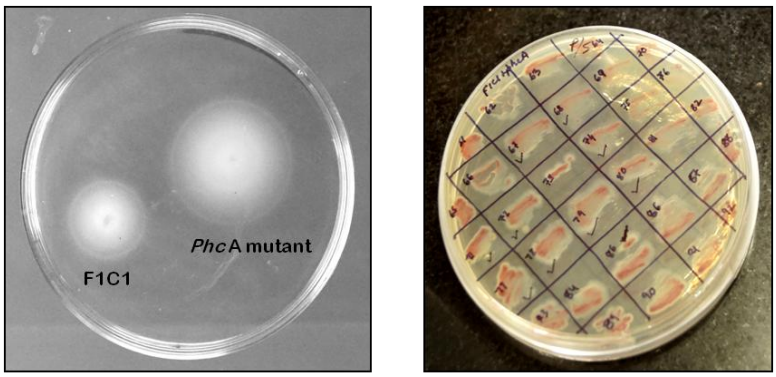


Fig.2.5: Colony phenotype of *phcA* mutant of *R. solanacearum* F1C1. The *phcA* mutant showed (A) hyper motility in soft agar plate in compared to wild type F1C1 and (B) EPS deficient reddish colony on BG-agar plate.

2.3.13 Creation of mCherry tagged *R. solanacearum* F1C1, *hrpB* and *phcA* mutant of F1C1 and their colonization study in eggplant and tomato seedlings

For studying colonization of F1C1 and derivative mutants in eggplant seedlings, fluorescence tagged F1C1 strains were created. To create mCherry (red fluorescence) tagged F1C1 strain, the plasmid pRCG_Pps-mCherry carrying a constitutive mCherry reporter was used [27]. We have isolated the plasmid from the DH5α strain harboring pRCG_Pps-mCherry plasmid using Qiagen plasmid isolation kit according to manufacturer's recommendations. Isolated plasmid was then linearized with restriction enzyme *ApaI*, confirmed by running on a 0.8% agarose gel and eventually the linear plasmid was naturally transformed into *R. solanacearum* F1C1 using the methodology as described above. mCherry labeled transformants appeared after 48 hr of incubation were selected on BG-agar plates containing gentamycin antibiotic.

For the creation of mCherry marked *hrpB* and *phc*A mutants of F1C1, genomic DNA was isolated from freshly grown TRS1012 and TRS1013 colonies cultured in BG broth using Qiagen genomic DNA isolation kit and was used to naturally transform mCherry-marked F1C1 named TRS1016. Transformants for both the types were selected on BG agar media supplemented with gentamycin and spectinomycin together after 48 hr incubation at 28ºC.

Saturated inoculums (~10^9 CFU/ml) of mCherry labelled F1C1 (TRS1016), *hrpB* mutant (TRS1017) and *phc*A mutant (TRS1018) of F1C1 were used for leaf

inoculation of eggplant (Devgiri) and tomato (Durga) seedlings. Mock inoculated seedlings of both the hosts with water were kept for control study.

After 4 DPI, infected seedlings were took out and washed well with sterile distilled water and subsequently dipped in 70% ethanol for 1-2 min for surface sterilization. Seedlings were then washed twice with sterile distilled water and were observed for red fluorescence under the fluorescence microscope (EVOS FL, Life technologies) equipped with 4X magnification.

2.3.14 Isolation of bacteria from the inoculated seedlings at different DPI

In order to estimate the bacterial load in the inoculated seedlings at different DPI and to propose a colonization pattern of F1C1 in eggplant and tomato we followed simple dilution plating method shown in picture below (Fig.2.6). For that, both 14 days old eggplant and 7 days old tomato seedlings were inoculated with TRS1016 at saturated concentration (~10^9 CFU/ml) by leaf clip inoculation method and bacterial load in the inoculated seedlings were counted at 0, 1, 2, 3, 4 days post inoculation (DPI). Two seedlings each were taken in a set for both tomato and eggplant and two replicates were considered for the experiment. The experiment was performed three times independently. At each DPI, both eggplant and tomato seedlings were taken out and surface sterilized with 70% ethanol followed by washing with sterile distilled water. Sterilized seedlings were homogenized in 1 ml of sterile distilled water separately using micro pestle (Abdos, India). Homogenates were further diluted with water and 100 µl homogenate was plated on BG-agar plate supplemented with gentamycin antibiotic at different dilutions for both the hosts. On 0 DPI, we plated 10^0, 10^2 fold, on 1 DPI 10^4, 10^5 fold, on 2 and 3 DPI 10^4, 10^5 and on 4 DPI 10^5,10^6 fold diluted homogenates and plates were incubated at 28°C for 48 hr. The number of colonies appeared in selected plates was recorded and colony number obtained at highest dilution in each day was considered for estimation.

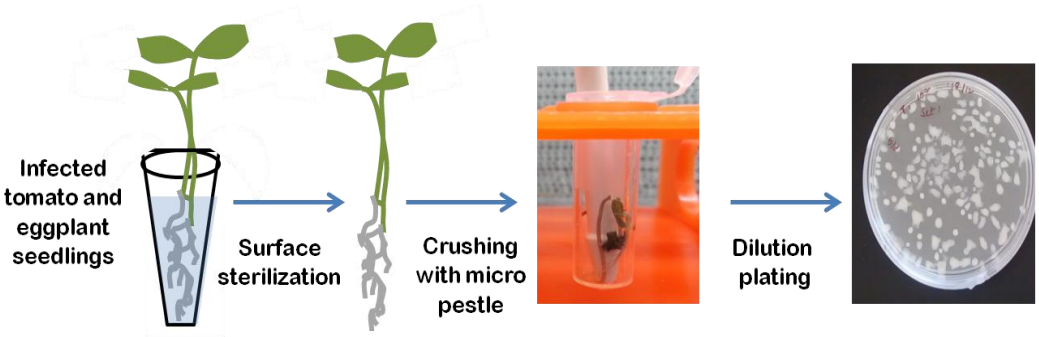


Fig.2.6: Schematic diagram depicting bacterial load count experiment. *R. solanacearum* infected tomato and eggplant seedlings inoculated by the leaf clip method were surface sterilized, crushed and homogenates were dilution plated to estimate the pathogen population.

2.4 Results

2.4.1. *R. solanacearum* F1C1 causes wilt in eggplant seedlings inoculated by the leaf clip method

We studied *R. solanacearum* F1C1 pathogenicity in eggplant by employing 15 days old early stages of eggplant seedlings by the leaf clip method under gnotobiotic conditions. Upon inoculation with saturated concentration of about ~10^9 CFU/ml, some of the inoculated seedlings started exhibiting the disease symptom after 2 days post inoculations. On 5 DPI, out of the 40 seedlings inoculated, more than 90% seedlings were found dead and by 7 DPI, almost all the inoculated seedlings were killed while in case of un inoculated control seedlings, no death of seedlings was observed as described in Fig.2.7 and Fig.2.8. The virulence was significantly drastic and could be observed at all times of inoculation by this method. We observed either no escapees or a few escapees in case of eggplant. Observation of either no escapees or very few escapees in case of eggplant seedlings was interesting because ~ 10% escapees were regularly observed in case of tomato seedlings inoculated with *R. solanacearum* by the leaf clip method.

To confirm that the eggplant susceptibility in this mode of inoculation is due to *R. solanacearum* pathogenicity, eggplant seedlings were inoculated with known non-pathogenic bacteria such as *E. coli* and *P. putida.* However, we did not observe any disease symptoms in these seedlings till 10^{th} DPI (Fig.2.8).

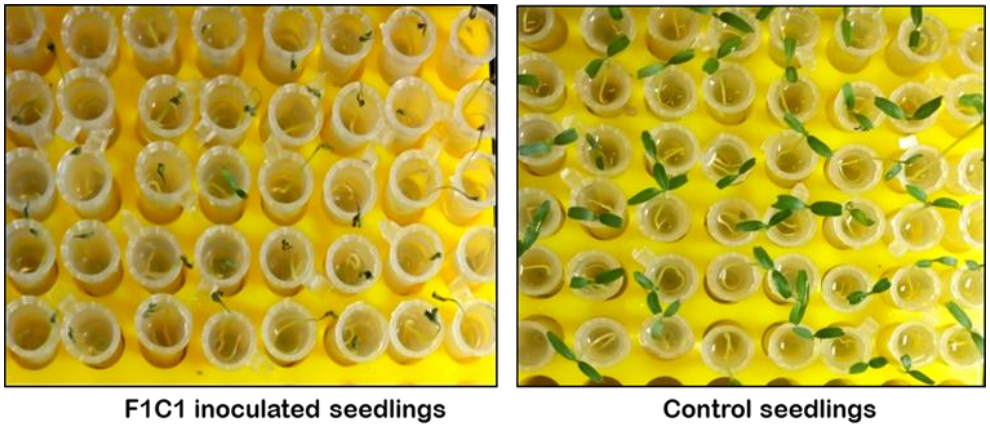


Fig.2.7: Representative picture depicting pathogenicity of *R. solanacearum* F1C1 in eggplant seedlings inoculated by the leaf clip method. All the seedlings inoculated with F1C1 were killed on 7th DPI while control seedlings mock inoculated with water were healthy.

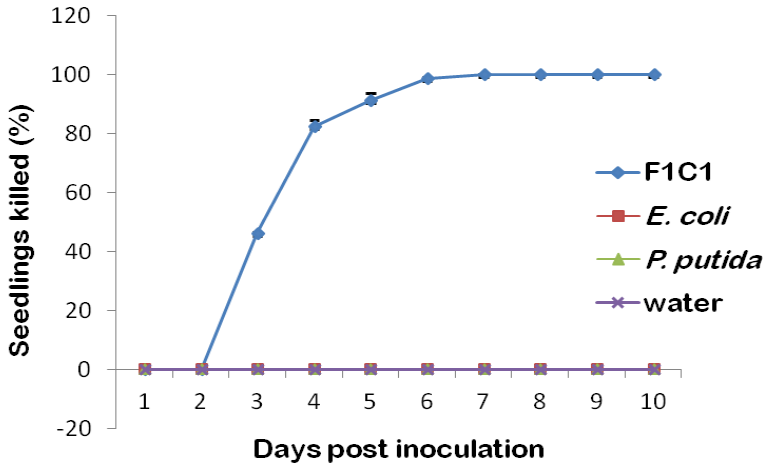


Fig.2.8: Pathogenicity study of *R. solanacearum* F1C1 and non-pathogenic bacteria in eggplant seedlings by the leaf clip inoculation. F1C1 was causing more aggressive wilting while there was no disease in eggplant seedling by non-pathogenic *P. putida* and *E. coli*. Each data point in this graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors.

We observed similar magnitude of pathogenicity of F1C1 studied in seedlings of two other eggplant cultivars; DevKiran and Param Hybrid. Although disease progression was different in all the three cultivars checked, by 10th DPI, F1C1 could kill all the seedlings of all the cultivars indicating general pathogenicity towards eggplants (Fig.2.9).

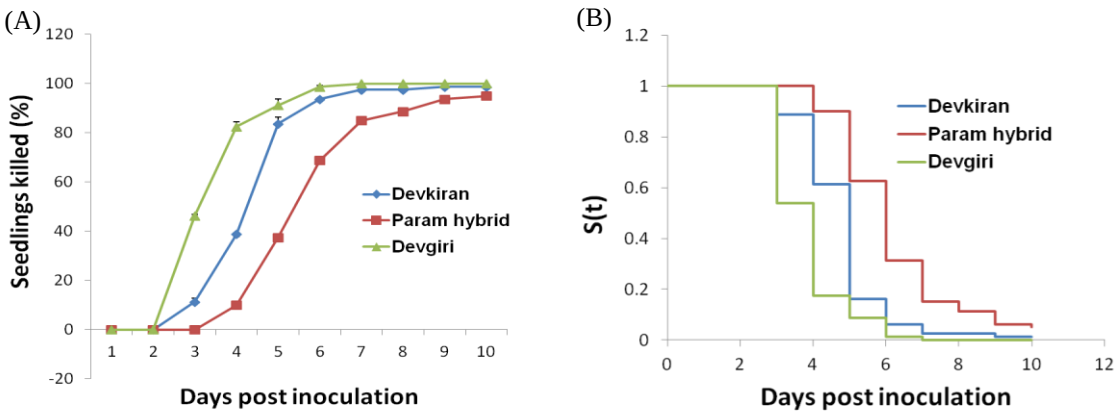


Fig.2.9: Pathogenicity of *R. solanacearum* F1C1 in different cultivars of eggplant. F1C1 (10^9 CFU/ml) were inoculated in seedlings of Devkiran, Param Hybrid and Devgiri by the leaf clip method and found to cause disease in all cultivars. (A) Line graph showing percentage of seedlings killed against DPI. Each data point in this graph is an average of three independent experiments with two replicates. Per replicate, 40 eggplant seedlings were inoculated. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings.

To check the pathogens minimum concentration that is sufficient to cause the disease in eggplant, we inoculated the eggplant seedlings with different concentration of the pathogen ranging from 10^9 CFU/ml to 10^3 CFU/ml. We observed almost similar magnitude of pathogenicity and disease progression in case of 10^9 and 10^7 CFU/ml concentrations. In case of 10^5 CFU/ml, number of seedlings died on 8 DPI was similar to the number of seedlings died on 5 DPI in case of 10^7 CFU/ml. In case of 10^4 CFU/ml, number seedlings died were significantly lower. At higher dilution, the magnitude of pathogenicity decreases as bacterial concentration in the inoculums decreases (Fig.2.10), which might be proportional to the number of initial bacteria deposited at the inoculated site. From our previous experience with tomato seedlings, the pathogenicity progression observed in eggplant indicated that *R. solanacearum* F1C1 is more aggressive in eggplant seedlings than tomato seedlings by the leaf clip inoculation.

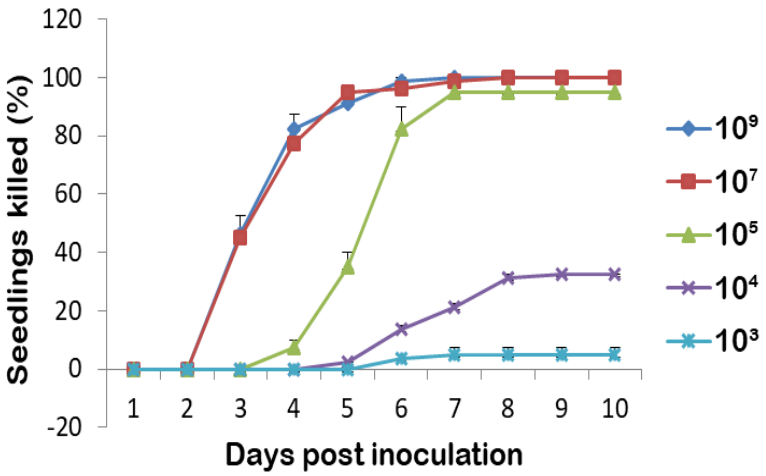


Fig.2.10: Pathogenicity assay with different concentration of F1C1 in eggplant seedlings. Wild type F1C1 of concentration ranging from 10^9 to 10^3 CFU/ml were inoculated in eggplant by the leaf clip method and observed that F1C1 can cause wilting up to 10^4 CFU/ml concentrations. Almost similar disease progression was observed in 10^9 CFU/ml and 10^7 CFU/ml concentrations while in 10^3 CFU/ml concentration, disease symptom observed was very minimal. Each data point in this graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors.

To confirm the association and colonization of F1C1 in eggplant seedlings inoculated by this method, we performed GUS staining of the infected eggplant seedlings. GUS staining was observed along the shoot region of the seedlings suggesting the growth and migration of the bacteria towards the root region along with the shoot (Fig.2.11a).

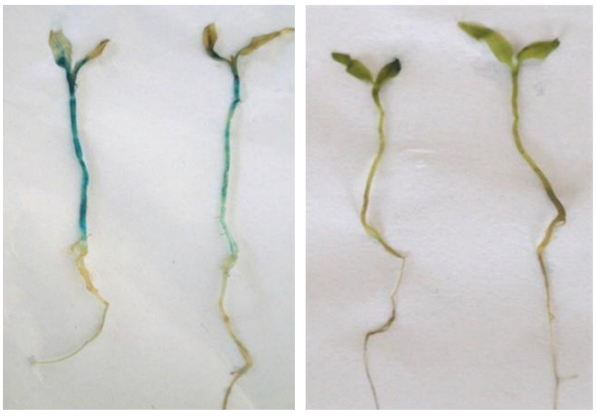


Fig.2.11a: GUS staining of infected eggplant seedlings inoculated by the leaf clip method. Infected eggplant seedlings inoculated with the *gus* tagged *R. solanacearum* F1C1 were examined on 4th day by X-gluc staining. (A) Blue staining in the dead infected seedlings

confirmed the bacterial colonization (B) Staining of control seedlings, without TRS1002 inoculation exhibited no blue colour.

We further confirmed the bacterial association in eggplant by fluorescence staining. We used mCherry marked F1C1 to inoculate the seedlings and observed pathogen colonization throughout the seedling (Fig.2.11b).

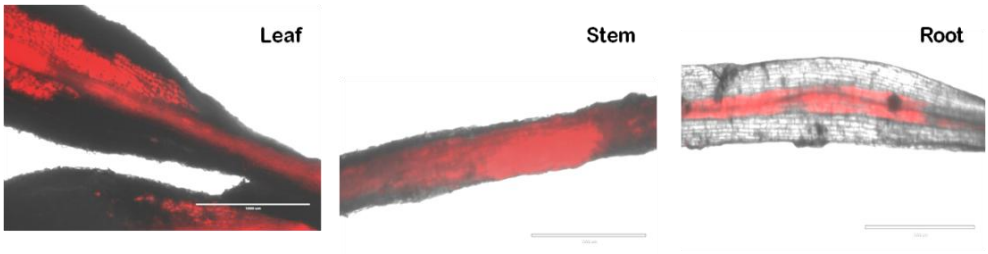


Fig.2.11b: Fluorescence staining of eggplant seedlings inoculated with mCherry tagged F1C1. Infected eggplant seedlings inoculated with *R. solanacearum* TRS1016 strain were examined on 4th day under fluorescence microscope. Red fluorescence in leaf, stem and root portion of the infected seedlings confirmed the bacterial colonization.

Since HrpB, HrpG and PhcA are well established transcription regulators of many pathogenicity determinants in this bacterium (Genin et al., 2005), we inoculated eggplant seedlings with *hrpB, hrpG* and *phcA* mutants to find out if the leaf clip inoculation method is efficient to study pathogenicity determinants of *R. solanacearum* F1C1. The *hrpB* and *hrpG* mutant were found to be non-pathogenic and the *phcA* mutant was found to be altered in its virulence in eggplant seedlings (Fig.2.12a). Surprisingly the magnitude of virulence deficiency in the *phcA* mutant was not high in eggplant seedlings in comparison to our earlier observation in the tomato seedlings [16]. This further indicated that *R. solanacearum* F1C1 was more aggressive in eggplant seedlings than tomato seedlings. The virulence data obtained were also analyzed by Kaplan-Meier survival statistics and statistical significance was calculated by log-rank test as shown in Fig.2.12b.

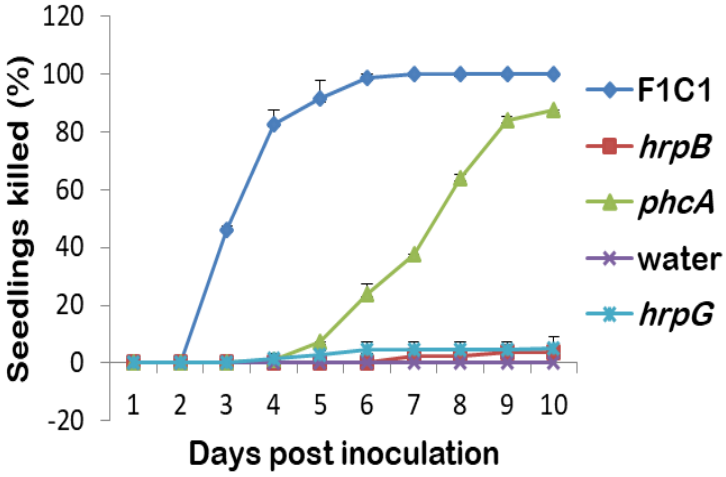


Fig.2.12a: Virulence of *R. solanacearum* F1C1 wild type, *hrpB, hrpG* and *phcA* mutants in eggplant seedlings. Eggplant seedlings were inoculated by the F1C1 wild type, *hrpB, hrpG* and *phcA* mutants with saturated concentrations (10^9 CFU/ml). By 7 DPI, all seedlings were killed in case of the F1C1 wild type. In case of *phcA*, till 4 DPI, almost all the seedlings were healthy, but by 9 DPI, more than 80 % seedlings were killed. In case of *hrpB* and *hrpB* mutants, by 9 DPI only 3% and 5% seedlings were found dead respectively. This suggested that *phcA* mutant is virulence deficient while *hrpB* and *hrpG* mutants are non-pathogenic. Each data point in this graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors.

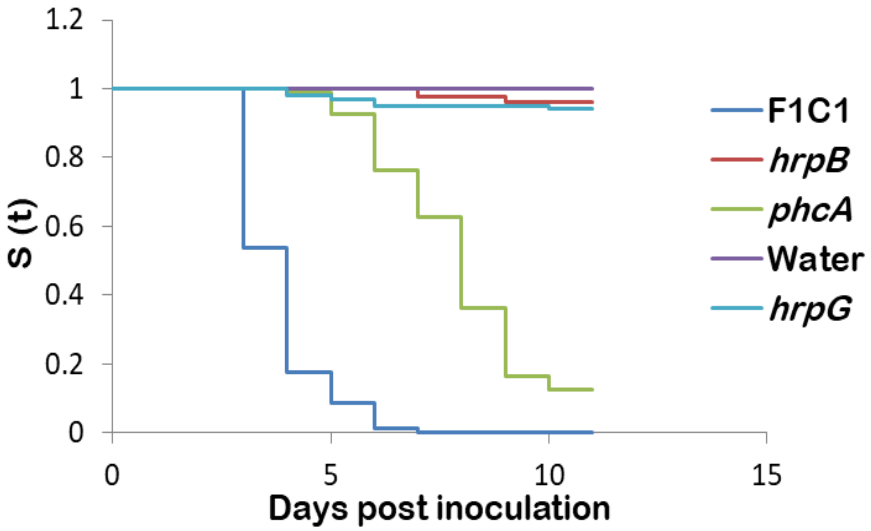


Fig.2.12b: Kaplan–Meier survival probability [S(t)] curve of eggplant seedlings inoculated with wild type F1C1 and derivative mutants. The *phcA* mutant of F1C1 exhibited significant virulence deficiency in eggplant seedlings in compared to the wild type F1C1 ($P < 0.001$; log-rank test), while *hrpB* and *hrpG* mutant was found to be non pathogenic in eggplant seedlings.

2.4.2 *R. solanacearum* F1C1 is more aggressive in eggplant seedlings than tomato seedlings inoculated by the leaf clip method

Though *R. solanacearum* is known to cause disease in many hosts, a comparative study on its pathogenicity between two hosts is not common. As described above, during the pathogenicity study of *R. solanacearum* in eggplant seedlings, we realized that F1C1 is more aggressive in eggplant in compared to tomato seedlings. This observation triggered us to do a comparative pathogenicity study between tomato and eggplant seedlings. For that, we recruited seedlings of both the hosts in a single microfuge tube and seedlings were inoculated with F1C1 wild type, *hrpB, hrpG* and *phcA* mutants by the leaf clip method. We observed more disease aggressiveness and faster disease progression in the eggplant seedlings inoculated with F1C1 and the *phcA* mutant than the tomato seedlings, inoculated with the same strains. However, *hrpB* mutant was found to be non- pathogenic in both the hosts (Fig.2.13a; Fig.2.13b). The *hrpG* mutant was non pathogenic in eggplant; however towards the later DPI it killed around 13% seedlings in tomato while it was 5% in case of eggplant. The virulence results were also analyzed by Kaplan-Meier survival statistics and statistical significance was calculated by log-rank test as shown in Fig.2.13c. The differential aggression of *R. solanacearum* in tomato and eggplant seedlings by leaf clip inoculation was very distinct.

Fig.2.13a: A representative picture showing differential pathogenicity of *R. solanacearum* between eggplant and tomato seedlings. Both eggplant and tomato seedlings recruited in a single microfuge tube were inoculated with (A) water (B) F1C1 wild type (C) *phcA* mutant (D) *hrpB* mutant and (E) *hrpG* mutant and observations were made each day. Picture was taken on 4 DPI. All eggplant seedlings were killed earlier than the tomato seedlings in case of F1C1 wild type. Similarly in case of *phcA* mutant, eggplant seedlings developed disease while tomato seedlings were healthy. In case of *hrpB* and *hrpG* mutants, both eggplant and tomato seedlings were healthy like the water inoculated seedlings.

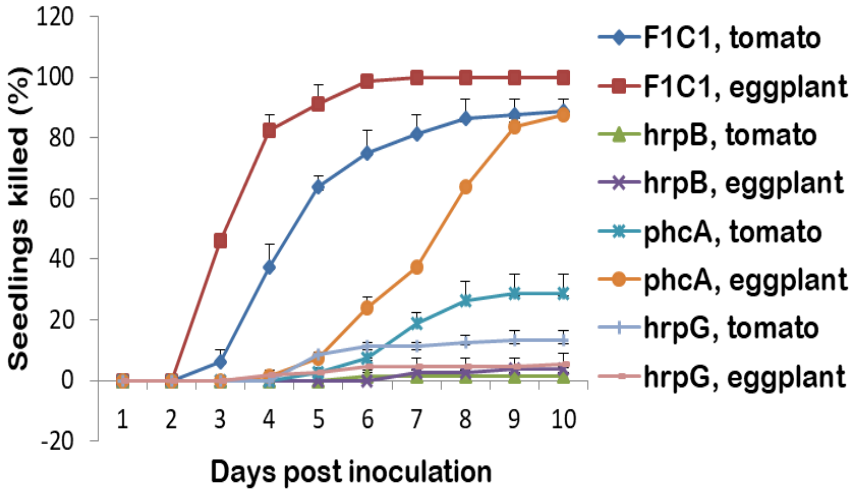
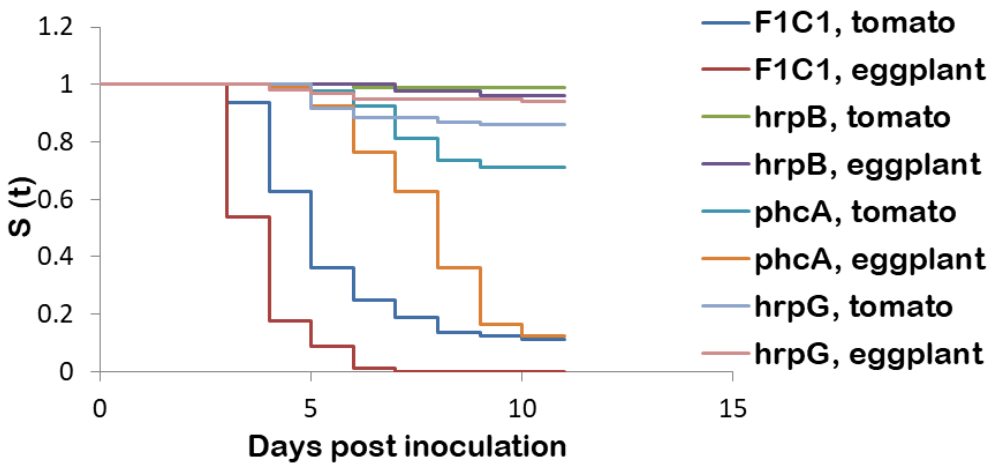


Fig.2.13b: Comparative virulence of *R. solanacearum* F1C1 wild type, *hrpB, hrpG* and *phcA* mutants between eggplant and tomato seedlings. Tomato and eggplant seedlings were inoculated with F1C1 and *phcA, hrpB* and *hrpG* mutants of F1C1 and both seedlings were put in a single microfuge tube to study *R. solanacearum* aggressiveness in the two host seedlings. Wild type F1C1 exhibited more disease aggressiveness in eggplant seedlings in comparison to tomato. Similarly, in case of *phcA* mutant, disease aggressiveness was more in eggplant after 6 days post inoculation than in tomato seedlings. However, *hrpG* was found to be slightly more aggressive in tomato seedlings than eggplant though it was much reduced for virulence. The *hrpB* mutant was nonpathogenic both in eggplant and tomato seedlings. Each data point in the line graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors.



Strain	Tomato/eggplant
F1C1	P<0.001
hrpB	P>0.1
hrpG	P>0.05
phcA	P<0.001

Fig.2.13c: Kaplan–Meier survival probability [S(t)] of eggplant and tomato seedlings inoculated with wild type F1C1, *hrpB, hrpG* and *phcA* mutants of F1C1 by the leaf clip method. Here, steep lines are clearly indicating that F1C1 is more aggressive in eggplant seedlings in compared to tomato seedlings. Similarly, *phcA* mutant is showing more disease aggressiveness in eggplant after 6 days post inoculation than in tomato seedlings whereas, *hrpB* mutant is non pathogenic in both eggplant and tomato seedlings. Though *hrpG* mutant was virulence deficient in both the hosts, it killed some seedlings more in tomato than eggplant unlike *phcA* mutant.

We further confirmed this phenotype of aggressive pathogenicity in eggplant seedlings by using 10^4 CFU and 10^5 CFU ml^{-1} concentration of *R. solanacearum* F1C1. F1C1 exhibited more disease aggressiveness in eggplant seedlings in comparison to tomato in both the concentrations. In case of 10^5 CFU ml^{-1} concentration, disease aggressive was more distinctly observed. Eggplant seedlings inoculated with 10^4 CFU ml^{-1} of pathogen exhibited more disease symptoms than tomato seedlings inoculated with 10^5 CFU ml^{-1} of the pathogen (Fig.2.14).

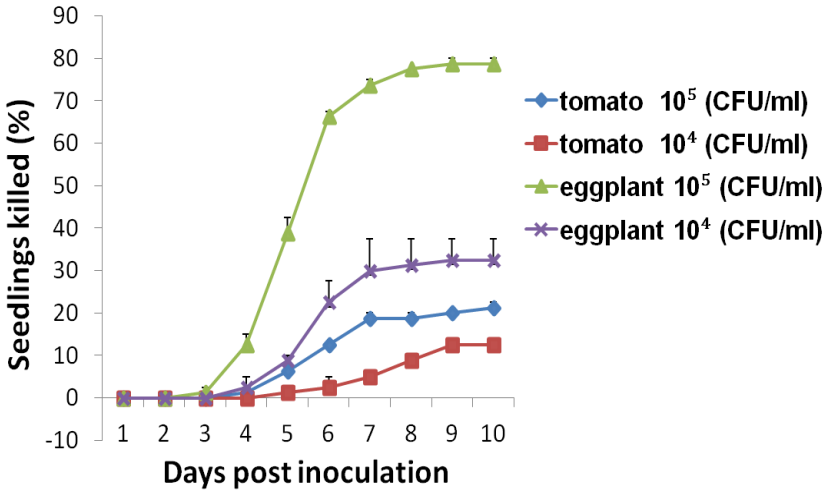


Fig.2.14: Comparative virulence of *R. solanacearum* F1C1 between eggplant and tomato seedlings in lower concentration. Tomato and eggplant seedlings incubated in the same tube were inoculated with F1C1 (10^5 CFU and 10^4 CFU/ml). In both concentrations, F1C1 exhibited more disease aggressiveness in eggplant seedlings in comparison to tomato. Each data point in the line graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors.

After observing differential pathogenicity of *R. solanacearum* between eggplant and tomato seedlings, we were interested to check the differential colonization if any of F1C1 and *hrpB* and *phcA* mutants of F1C1 in eggplant and tomato seedlings. For that we used mCherry tagged strains of F1C1 (TRS1016), *hrpB* (TRS1017), and *phcA* (TRS1018) and inoculated by the leaf clipping. We observed colonization and spreading of F1C1 in leaf, stem as well as in root regions both in case of tomato and eggplant seedlings. Colonization was observed to be more in eggplant seedling than the tomato seedling though we have not quantified the fluorescence. Similar pattern was found in case of the *phcA* mutant inoculated seedlings of eggplant and tomato, however colonization was lesser in compared with wild type F1C1. In case of *hrpB* mutant, the migration and colonization was observed to be largely restricted to the inoculated leaf areas of both eggplant and tomato seedlings indicating the growth deficiency of *hrpB* mutant inside the seedlings. However, we observed more fluorescence in eggplant leaf compared to tomato leaf. Results have been shown in Fig.2.15a and Fig.2.15b.

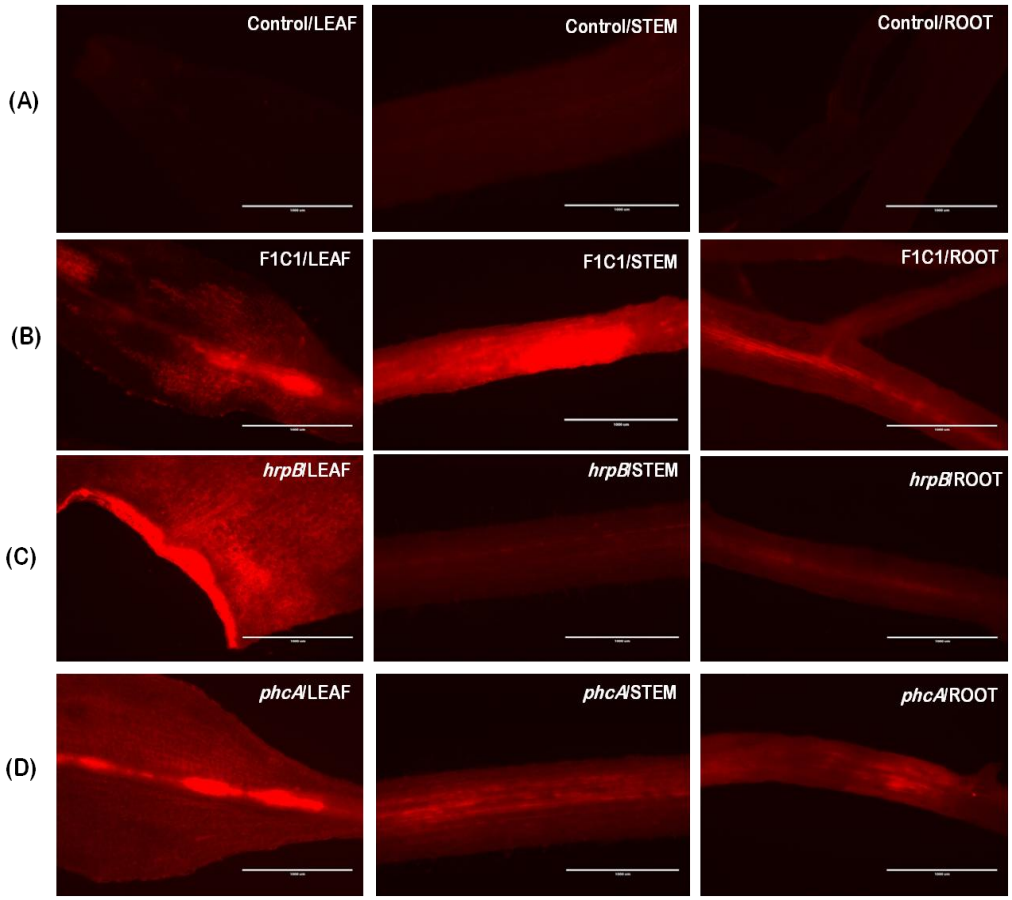


Fig.2.15a: Fluorescence staining of eggplant seedlings inoculated with F1C1 and derivative mutants. Eggplant seedlings inoculated with (A) Water (B) mCherry tagged *R. solanacearum* (TRS1016) (C) *hrpB* mutant (TRS1017) and (D) *phcA* mutant (TRS1018) were observed under the fluorescence microscope on 4 DPI. In case of TRS1016, fluorescence was observed in leaf, stem as well as in root regions, whereas in case of TRS1017 fluorescence was largely limited to the region in the leaf where inoculation was done and was very faint in stem and root regions. In case of TRS1018, fluorescence was observed in leaf, stem and root like TRS1016.

In another study, we investigated the colonization of *hrpB* mutant in eggplant as well as in tomato seedlings after co inoculation with F1C1 wild type in 1:1 ratio. Interestingly, we found that *hrpB* mutant can migrates from the inoculation sites to the roots of the seedlings when co inoculated with the wild type (Fig.2.15c).

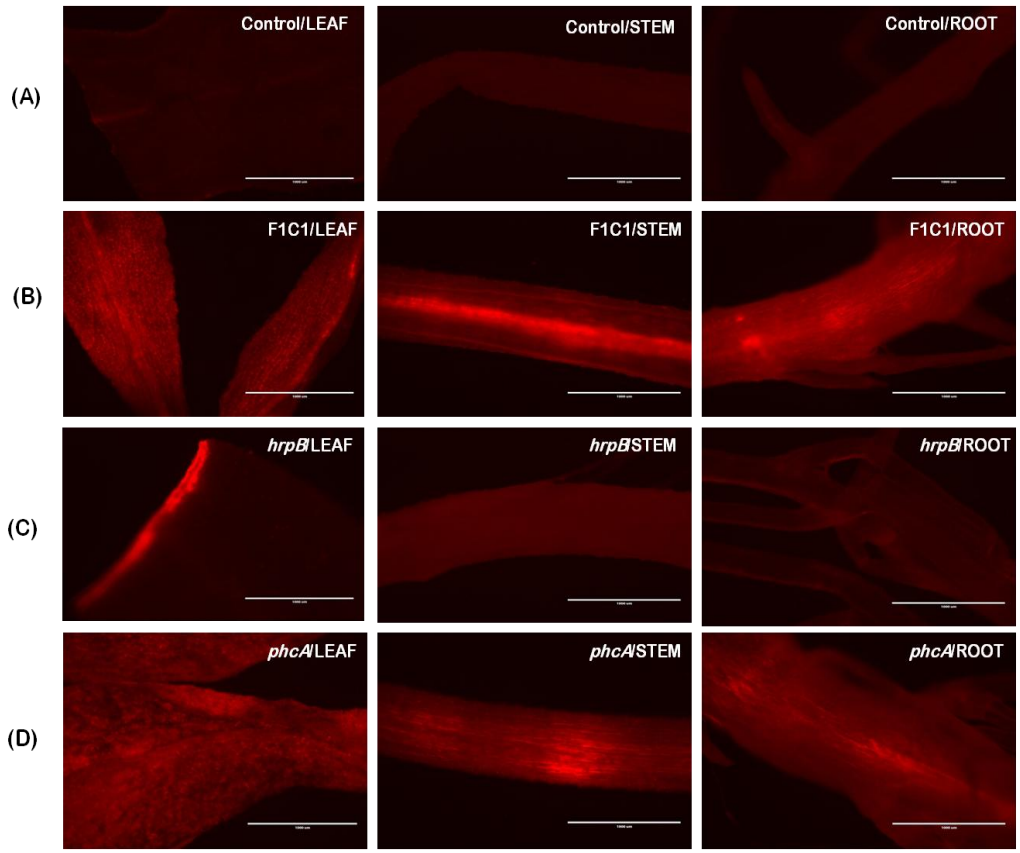


Fig.2.15b: Fluorescence staining of tomato seedlings inoculated with F1C1 and derivative mutants. Tomato seedlings inoculated with (A) Water (B) mCherry tagged *R. solanacearum* (TRS1016), (C) *hrpB* mutant (TRS1017) and (D) *phcA* mutant (TRS1018) were observed under the fluorescence microscope on 4 DPI. In case of TRS1016, fluorescence was observed in leaf, stem as well as in root regions, whereas in case of TRS1017 fluorescence was largely limited to the region in the leaf where inoculation was done and was very faint in stem and root regions. In case of TRS1018, fluorescence was observed in leaf, stem and root like TRS1016.

Since, a difference in magnitude of virulence of *R. solanacearum* between eggplant and tomato seedlings was observed; we determined the colonization in terms of bacterial load both in eggplant as well as tomato seedlings. We inoculated TRS1016 (10^9 CFU/ml) in the seedlings of both the hosts by the leaf clip method and bacterial colony forming unit (CFU/ml) was calculated for 5 subsequent days post inoculation (0, 1, 2, 3 and 4 DPI) (Fig.2.16).

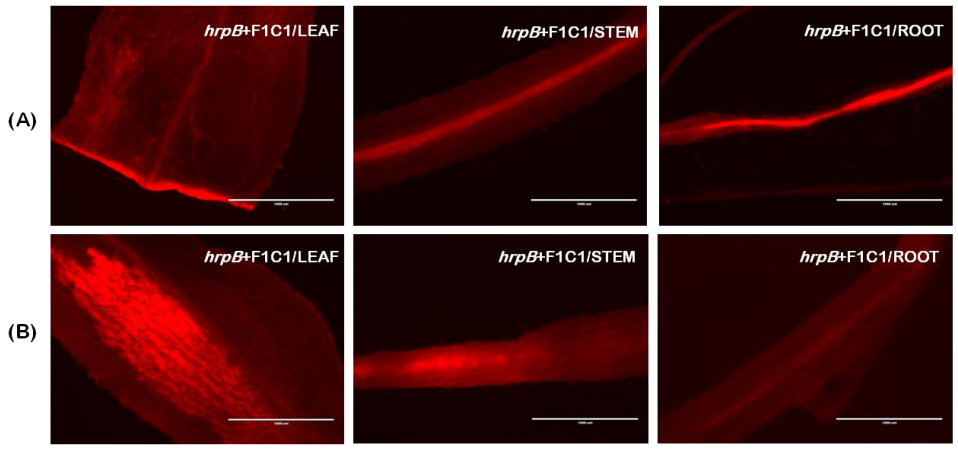


Fig.2.15c: Fluorescence staining of seedlings co-inoculated with F1C1 and *hrpB* mutant. (A) Tomato and (B) Eggplant seedlings were inoculated with mCherry tagged *hrpB* mutant (TRS1017) and wild type F1C1 together (1:1) by the leaf clip method. Infected seedlings of both hosts were observed under the fluorescence microscope and red fluorescence was observed in leaf, stem as well as in root regions, suggesting co-migration of *hrpB* mutant along with wild type F1C1 in seedlings.

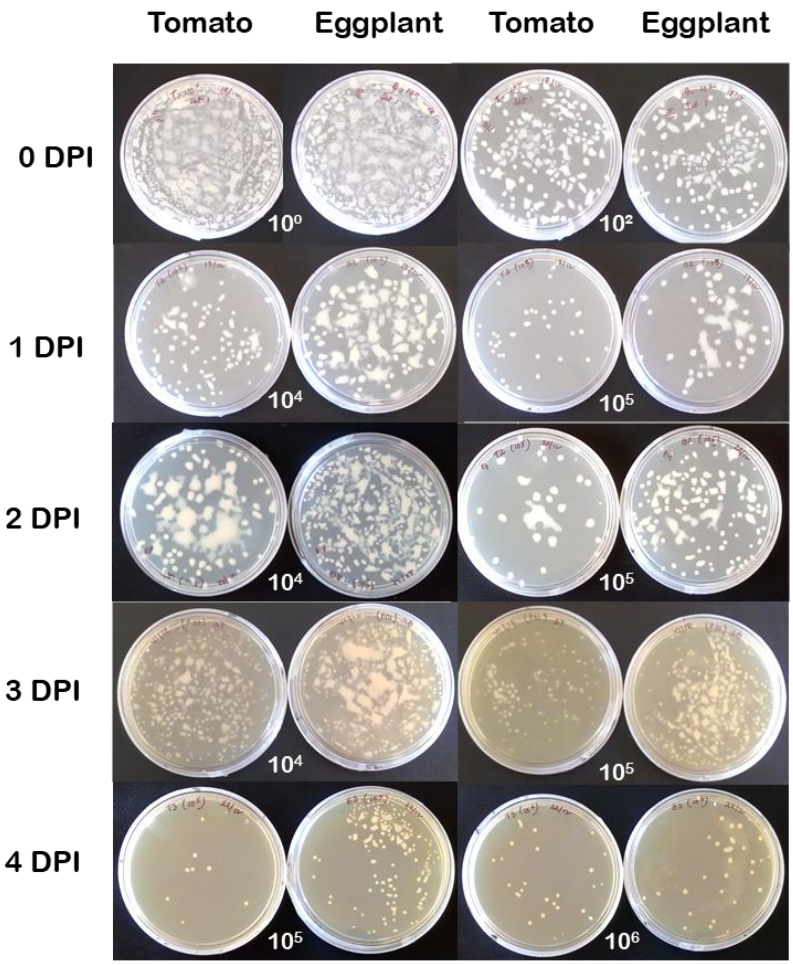


Fig.2.16: Representative picture showing bacterial colonies in plates in infected eggplant and tomato seedlings inoculated with *R. solanacearum*. Eggplant and tomato seedlings were inoculated with saturated concentration (10^9 CFU/ml) of mCherry tagged F1C1 strain (TRS1016). At 0, 1, 2, 3 and 4 days post inoculations, the inoculated seedlings were individually surface sterilized, crushed and dilution plated. Colonies appeared were more in eggplant than tomato seedlings.

It was notable to observe that in both eggplant and tomato seedlings, *R. solanacearum* load were increased with days post inoculation till 4 DPI. Interestingly, in case of infected eggplant seedlings we observed that the pathogen concentration was higher than the infected tomato seedlings (Fig.2.17).

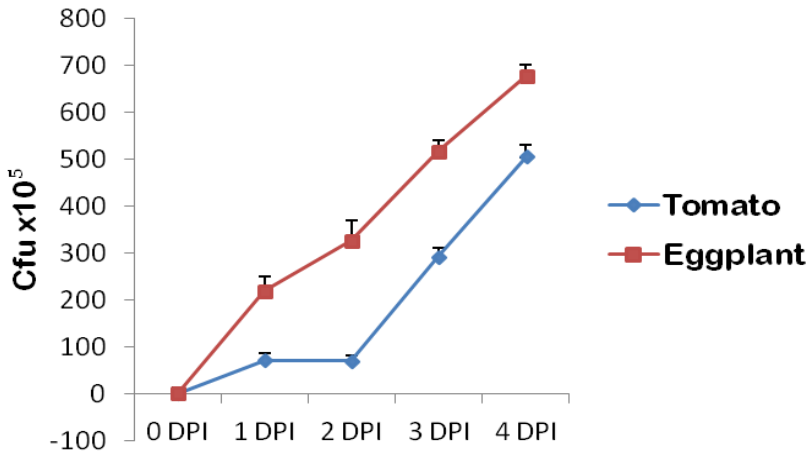


Fig.2.17: Comparative bacterial population in infected eggplant and tomato seedlings inoculated with *R. solanacearum*. Eggplant and tomato seedlings were inoculated with mCherry tagged F1C1 (TRS1016) with saturated concentration (10^9 CFU/ml). Starting from the day of inoculation (0 DPI) till 4 DPI, the inoculated seedlings were individually surface sterilized, crushed and dilution plated. X-axis presents DPI and Y-axis presents CFU/ml. Colonies (CFU x10^5/ml) observed were increasing with days up to 4 DPI. Though on zero DPI, bacterial load was almost similar, in subsequent days it was more in infected eggplant seedlings in comparison to tomato seedlings. Each data point in this graph is an average of three independent experiments with two replicates.

2.5 Discussion

In this study, we demonstrated that the leaf clip inoculation is a stable and consistent method to study *R. solanacearum* pathogenicity in two leaves cotyledon stage of eggplant seedlings. Some of the known virulence deficient mutants could be differentiated according to their pathogenicity behavior in eggplant seedlings. Then, by recruiting early stages of seedlings of tomato and eggplant within a single

microfuge tube we have demonstrated that *R. solanacearum* is more aggressive in eggplant seedlings than tomato seedlings. This conclusion is based on the following observations made in this study such as (i) percentage of eggplant seedlings died was always more than that of the tomato seedlings when inoculated with the same bacterial load; (ii) the disease appeared more rapidly in the eggplant seedlings than the tomato seedlings inoculated with same bacterial load; (iii) the *phcA* mutant was found to be more aggressive in eggplant seedlings than in tomato seedlings; and (iv) the bacterial load in the inoculated eggplants seedlings were higher than that in the tomato seedlings after two DPI onwards. These all indicate that the adaptation of the bacterium in two different hosts is not alike: it is likely to be better in eggplant seedlings than that in the tomato seedlings by the leaf clip inoculation method. We believe that this is the first demonstration of the *R. solanacearum* comparative pathogenicity in two different hosts maintained in close proximity.

The higher pathogenicity of *R. solanacearum* in eggplant seedlings in comparison to tomato seedlings by the leaf clip inoculation might be argued in favor of the genotypes used in the respective hosts rather any attribution to the hosts *per se*. Though the above possibility can't be ruled out, we have the following reasons to believe that *R. solanacearum* pathogenicity in general is higher in eggplant seedlings than the tomato seedlings by the leaf clip inoculation. Previously in our laboratory we had evaluated *R. solanacearum* pathogenicity in seedlings of eight different commercially available tomato cultivars (Kumar et al., 2017, *plant pathology*) where the cultivars exhibited different magnitude of disease susceptibility towards the pathogen, amongst which Durga cultivar exhibited highest susceptibility, which has been used in this study while comparing with the pathogenicity with eggplant seedlings. In all the tomato cultivars, escapees were observed. In addition, in comparison of one leaf clip and two leaves clip inoculation in different tomato cultivars always produced higher escapees in one leaf clip inoculation than the two leaves clip inoculation. The three eggplant cultivars recruited in the present study exhibited equal susceptibility among each other with regard to *R. solanacearum* pathogenicity and higher susceptibility than the tomato seedlings. Moreover, when we studied one leaf and two leaves clip inoculation in eggplant seedlings, in both the cases we observed ~100% wilting (unpublished data from the laboratory).

How the pathogen's aggressiveness is different between the two susceptible hosts is an obvious question emerged from this study. It is pertinent to note that the experiment has been conducted in the seedlings stages where in comparison to grown up plants, the two seedlings are in their early development and expected to be in a more susceptible stage. Further the leaf clip inoculation method allows the direct deposit of the bacterial inoculum at the cut end surface of the cotyledon leaves that avoids the complex root entry method in the pathogen infection. These arguments indicate the role of host's inside ambience influencing the pathogenesis process. Differential expression of *R. solanacearum* virulence functions in laboratory and in plants environments is known [28,29,30,31,32]. The bacterium uses an elaborate sensory and regulatory network involving many genes to regulate different virulence and pathogenicity factors [33]. In this study, we have used three regulatory mutants such as *hrpB, hrpG* and *phcA* to study their pathogenicity in eggplant in comparison to tomato. The *hrpB* mutant was found to be non-pathogenic in both hosts. It is reported that HrpB regulates the type III secretion system of *R. solanacearum* that is known to be an essential pathogenicity determinant in this pathogen [34]. The *hrpG* was found to cause wilt in a few seedlings in both hosts unlike *hrpB*, though HrpG positively regulates HrpB [34]. Surprisingly, the *phcA* mutant was found to be much more aggressive in eggplant seedlings in comparison to that in tomato seedlings. PhcA, has been described as the largest regulon in *R. solancearum* that is involved in the regulation of unusually a large number of genes (~ 30% genes in the genome) including important pathogenicity determinants such as exopolysaccharides, extracellular enzymes, motility and T3SS in this pathogen [31]. *In planta* gene expression study in tomato has revealed that *PhcA* is an important regulator for the strategic switch between attachment/spread and growth/virulence in this pathogen [29]. Therefore, its differential virulence behavior in the two hosts indicates that factors associated with *PhcA* may be contributing differently towards the pathogen adaptation inside different hosts. It also indicates that the conclusion derived regarding a pathogenicity function in respect to one host may not be fully true with respect to another host. In future, *in planta* gene expression studies in this pathogen with regard to different hosts will provide insight into the mechanism of virulence of the *phcA* mutant in eggplant seedlings.

R. solanacearum pathogenicity study in host plants is not so simple, because sometimes the bacterium remains associated with its hosts without wilting it [8,35]. Moreover, it has been observed that in a host, the disease aggressiveness varies depending on the mode of inoculation and it may vary from host to host. In our study, the leaf clip inoculation method effective in tomato was also effective in eggplant seedlings but, root inoculation method effective in tomato seedlings [36] was not effective in a same way for eggplant seedlings (data not shown). Though, it is known that the natural mode of entry of this pathogen is through root of its host, looking at the vigorous symptom developed in the seedlings upon leaf clip inoculation in two different hosts, under natural condition, *R. solanacearum* entry into its host by other means can't be eliminated. Like tomato, bacterial wilt is also a very common disease in eggplant. In spite of this, detail understanding of molecular mechanisms of disease on eggplant is still incomplete hence further studies will be of importance. The leaf clip method described here is quite simple and consistent and thus likely to be useful to study eggplant and *R. solanacearum* interaction.

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