An Innovative Root Inoculation Method to Study *Ralstonia solanacearum* Pathogenicity in Tomato Seedlings

N. Singh[1], T. Phukan[1], P. L. Sharma[1], K. Kabyashree[1], A. Barman[1,2], R. Kumar[1,3], R. V. Sonti[4], S. Genin[5], and S. K. Ray[1*]

[1]Dept of Molecular Biology and Biotechnology, Tezpur University, Tezpur–784028, Assam, India

[2]Present address: Department of Biotechnology, Pandu College, Guwahati-781012, Assam, India

[3]Present address: National Institute of Plant Genome Research, Aruna Asaf Ali Road, New Delhi – 110067, India.

[4]Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad–500007, Andhra Pradesh, India.

[5]LIPM, Université de Toulouse, INRA, CNRS, F–31326 Castanet-Tolosan, France.

*Corresponding author: Suvendra Kumar Ray

Email: suven@tezu.ernet.in

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ABSTRACT

In this study, we are reporting *Ralstonia solanacearum* pathogenicity in the early stages of tomato seedlings by an innovative root inoculation method. Pathogenicity assays were performed under gnotobiotic conditions in microfuge tubes by employing only 6-7 days old tomato seedlings for root inoculation. Tomato seedlings inoculated by this method exhibited the wilted symptom within 48 h and the virulence assay can be completed in two weeks. Colonization of the wilted seedlings by *R. solanacearum* was confirmed by using gus staining as well as fluorescence microscopy. Using this method, mutants in different virulence genes, such as *hrpB, phcA,* and *pilT*, could be clearly distinguished from wild-type *R. solanacearum.* The method described here is economic in terms of space, labor, cost as well as the required quantity of bacterial inoculum. The newly developed assay is thus an easy and useful approach for investigating virulence functions of the pathogen at the seedling stage of hosts and infection under these conditions appears to require pathogenicity mechanisms used by the pathogen for infection of adult plants.

INTRODUCTION

Ralstonia solanacearum is a Gram-negative plant pathogenic bacterium that causes a lethal wilt disease in more than 450 plant species belonging to several monocotyledonous as well as dicotyledonous plant families (Hayward, 1991; Elphinstone, 2005; Wicker et al., 2007; Genin, 2010). The pathogen exceptionally has a wide host range (Coutinho et al., 2000; Ozaki & Watabe, 2009; Jiang et al., 2016; Weibel et al., 2016). Several strains of the bacterium have been documented from different parts of the globe supporting adaptability of the pathogen to wide geographical ranges. Prevalence of noticeable genetic diversity across different strains of the

pathogen has led to coining of the term "species-complex" in regard to this pathogenic bacterium (Fegan & Prior, 2005). Owing to the lethality it causes to its numerous hosts, it was ranked the second most devastating bacterial phytopathogen in the world (Mansfield et al., 2012).

R. solanacearum dwells in soil. In the presence of a host plant, the bacterium attaches to its root, enters the plant, grows and colonizes inside the xylem, subsequently resulting in the wilting and killing of the plant (Genin, 2010). Several important regulatory networks as well as involvement of different protein secretion systems (such as type II & type III) essential for virulence functions in this bacterium have been uncovered (Vasse et al., 2000; Genin & Boucher, 2002; Monteiro et al., 2012a; Coll & Valls, 2013). The *in planta* gene expression studies of *R. solanacearum* (Jacobs et al., 2012; Puigvert et al., 2017; Ferreira et al, 2017) have provided several clues on the adaptive responses of the bacterium within its host. Recently the role of diffusible quorum sensing molecules, extracellular DNAses as well as biofilm formations were reckoned to be important for its pathogenic interaction with host (Kai et al., 2015; Tran et al., 2016; Mori et al., 2016; Hikichi et al., 2017). Despite wealth of exciting findings in regard to *R. solanacearum* biology (Marchetti et al, 2010; Remigi et al., 2014; Guidot et al., 2014; Peyraud et al., 2016; Hikichi et al., 2017), knowledge pertaining to mechanism of its entry into host, *in planta* growth and multiplication, and the factors that trigger bacterium's pathogenicity functions within the host are incomplete. It is also unknown whether the pathogen discriminates between seedling stages and adult stages of its host in natural conditions.

R. solanacearum is referred as an attractive model for investigating fundamental aspects in plant-pathogenic bacteria interaction as well as pathogen-host adaptations (Genin & Boucher, 2002; Genin, 2010; Coll & Valls, 2013; Ferreira et al, 2017). Grown up tomato plants are generally utilized as suitable model host where the pathogen is inoculated either by soil

drenching or by stem inoculation. Besides, *Arabidopsis thaliana, Phaseolus vulgaris* (a distant host plant), *Mimosa pudica* (a non-host plant) are other model plants which have been utilized for understanding virulence, host resistance and evolution of *R. solanacearum* (Yang & Ho, 1998; Deslandes et al., 2002; Marchetti et al., 2010; Guidot et al., 2014). Host plants raised in soil comes in contact with soil-borne microbes and these associations in turn could modulate their fitness (Feau and Hamelin, 2017). Resident microbial population can therefore affect inferences of *R. solanacearum* virulence assays conducted on such hosts. Further, growing and maintaining large numbers of grown up plants requires ample amount of space, time as well as economic investments. In certain cases, soil drenching and stem inoculation methods were also not found appropriate for analyzing minute virulence differences in few mutants of *R. solanacearum* (Macho et al., 2010).

Seedling stages of tomato plants have been in use for studying *R. solanacearum* pathogenicity in number of occasions (Pradhanang et al., 2000; Park et al., 2007; Artal et al, 2012; Monteiro et al., 2012a; Kumar, 2014; Kumar et al., 2017). In fact there is an interesting recent report on *R. solanacearum* root infection in early stages of *Arabidopsis thaliana* under gnotobiotic condition (Lu et al.,2017). A gnotobiotic condition for *R. solanacearum* inoculation into tomato seedlings by leaf-clipping was recently documented from the author's laboratory (Kumar et al., 2017). Since *R. solanacearum* naturally infects its plant host *via* root, its behavior inside the plant may distinctly differ when the pathogen is introduced through the other means such as leaf clipping. Therefore, devising an efficient root inoculation method devoid of such constraints was imperative. In this context, here we are reporting an innovative method of *R. solanacearum* root inoculation into 6-7 days old tomato seedlings which is equally efficient in causing disease in different cultivars of tomato. The method has been successfully implemented

in studying pathogenicity functions of *R. solanacearum* by recruiting mutant strains for important virulence regulators such as *hrpB* and *phcA* in tomato seedlings. The method described here is reasonably rapid, easy, and cost effective as well as requires less inoculum of bacteria. Considering these attributes, this method of inoculation is anticipated to help significantly in understanding intricate molecular mechanisms of *R. solanacearum* virulence in seedling stages of hosts in near future.

MATERIALS AND METHODS

Bacterial strains, growth media and culture conditions. Bacterial strains used in the entire work have been listed in Table 1. Growth medium used for the wild type *R. solanacearum* F1C1 (Kumar et al., 2013), derivative mutant strains as well as *Pseudomonas putida* was BG medium (Boucher et al., 1985) supplemented with 0.5% glucose. Incubation temperature for *R. solanacearum* strains and *P. putida* was 28°C. *Escherichia coli* and *Bacillus subtilis* strains were grown in LB medium (Bertani, 1952) at 37° C. 1.5% agar was added in case of solid medium as and when necessary. Concentrations of different antibiotics used were as follows: spectinomycin (Spc; 50µgml^{-1}), ampicillin (Amp; 50µgml^{-1}), rifampicin (Rif; 50µgml^{-1}) and gentamycin (Gen; 50µgml^{-1}). All media components, antibiotics used in this work were bought from Himedia (Mumbai, India).

Germination of tomato seedlings for inoculation. Tomato seeds of different cultivars recruited in this study were pre-soaked in sterile distilled water for two days. This was followed by spreading the seeds on sterile wet tissue paper in a plastic tray and allowed to germinate in a growth chamber (Orbitek, Scigenics, India) maintained at 28°C, 75% relative humidity (RH), 12 h photo period respectively. Sterile distilled water was sprinkled regularly to sustain the

116 germination process till 6-7 days. Age of the seedling was defined from the day the seeds were
117 kept for germination on the wet tissue paper bed.

118 **Preparation of bacterial inoculum.** *R. solanacearum* F1C1 was streaked on BG-Agar plate.
119 Freshly grown *R. solanacearum* (F1C1) colonies were added to 50 ml BG broth with a sterile
120 loop and allowed to grow in a shaking incubator (Orbitek, Scigenics, India) maintained at 28°C
121 and 150 rpm. After 24 hours, bacterial cultures were centrifuged at 4000 rpm (3155 *g*) for 15 min
122 at 4°C. Bacterial pellets were resuspended in equal volume of sterile distilled water to obtain a
123 concentration of ~10^9 CFUml^{-1}. *P. putida* was grown in BG broth similar to *R. solanacearum* at
124 28°C while LB broth was used for culture of *E. coli* and *B. subtilis* at 37°C in a shaking
125 incubator maintained at 150 rpm. All the inoculums were prepared following the same procedure
126 used for *R. solanacearum* F1C1 mentioned above.

127 **Root inoculation of *R. solanacearum* in tomato seedlings.** About 15-20 ml of *R. solanacearum*
128 F1C1 inoculum (~ 10^9 CFU ml^{-1}) was taken in a sterile container (Fig. 1a; Fig. 1b). From the
129 germinated seedling tray, 6 -7 days old tomato seedlings were picked one at a time. Root of each
130 seedling was then dipped in the bacterial inoculum (up to the root-shoot junction) followed by
131 transfer of the seedling to an empty 1.5 or 2.0 ml sterile microfuge tube. All the seedlings were
132 inoculated by the same procedure. The root-dip inoculated seedlings transferred to microfuge
133 tubes were subjected to air exposure for ~5 minutes prior to addition of 1-1.5 ml of sterile water
134 to each tube. Exposure of the inoculated roots to air at this step was found to be critical for *R.*
135 *solanacearum* pathogenicity in tomato seedlings.

136 In all the experiments, set of minimum 40 (forty) seedlings were taken for each bacterial
137 inoculum used. In control set, 40 (forty) seedlings were mock-inoculated with sterile distilled
138 water following the same steps as mentioned above. All the Inoculated seedlings along with

controls were transferred to growth chamber maintained at 28°C, 75% RH, 12hrs photoperiod. Seedlings were analyzed for disease progression next day onwards till 7th day post-inoculation and findings were recorded.

Seedlings of four commercially available tomato cultivars namely Akhilesh (Param Hybrid Seeds), Vijay (Param Hybrid Seeds), Durga (selection -22) and Durga (ruby) were tested for *R. solanacearum* pathogenicity by the root inoculation method as described above.

Root inoculation of non-pathogenic bacteria such as *B. subtilis*, *P. putida*, *E. coli* in tomato seedlings was also done as above.

Root inoculation of tomato seedlings with different titers of *R. solanacearum* F1C1. Seven days old tomato seedlings of Durga cultivar (selection -22) were inoculated with different dilutions (10^0 to 10^6) i.e. 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 CFU ml^{-1} inoculum respectively of *R. solanacearum* F1C1 by the root inoculation technique described above to determine the effect of different titers of pathogen on disease progression. Set of forty seedlings were recruited in each dilution inoculation. Bacterial pelleting was performed as stated in previous sections. The resuspended bacterial pellets were serially diluted to obtain ~10^9 to ~10^3 CFU ml^{-1} of *R. solanacearum* (F1C1) by adding sterile distilled water prior to inoculation. Seedlings were analyzed for disease progression till the seventh day post inoculation and data was recorded.

Transformation in *R. solanacearum* F1C1 strain. The protocol used to transform F1C1 followed the method described by Gonzalez et al., (2011) with modifications in glycerol concentration used. F1C1 was grown in minimal medium containing 10% glycerol as a sole carbon source for 48 hrs at 28ºC. 50 µl of the bacterial inoculum was mixed with 1-2 µg of linearized genomic DNA as well as plasmid DNA and spotted on 0.45µm sterile nitrocellulose membrane kept over BG-agar plate without glucose. After 48 hrs of incubation at 28ºC, bacteria

were scooped out, resuspended in 150 µl of distilled water and plated on the selection medium containing appropriate antibiotics.

Creation of mCherry-tagged *R. solanacearum* F1C1 and its colonization study in tomato seedlings. The plasmid pRCG_Pps-mcherry carrying a constitutive mCherry gene expression reporter (Capela et al, 2017; Monteiro et al., 2012b) was linearized with restriction enzyme *ApaI* and naturally transformed into *R. solanacearum* F1C1. mCherry labeled transformants were selected on BG-agar plates containing gentamycin antibiotic. One of the mCherry labeled strain TRS1016 was cultured in BG broth containing appropriate antibiotic. TRS1016 culture was pelleted down and 10^9cfu/ml inoculum of the former was prepared by same method described for R. solanacearum above. TRS1016 inoculum was used for root inoculation of seven days old tomato seedlings as stated above. After three days post inoculation tomato seedlings were surface sterilized following method of Kumar et al., (2016). Sterilized seedlings were observed under the fluorescence microscopy (EVOS FL, Life technologies) in 40X magnification adjusted in RFP filter.

Creation of *pilT and rpoN2* mutants of *R. solanacearum* F1C1. Taking the reference genome of GMI1000 from LIPM database (https://iant.toulouse.inra.fr/), primers were designed for partial amplification of *rpoN2* and *pilT* gene homologs in *R. solanacearum* F1C1 strain. In all the sets of primers designed, forward primers contained a *Hin*dIII restriction site and the reverse primers were incorporated with *Xba*I restriction site at their 5' ends respectively. Primers sequences are given in Stable 8. Primers (5'-GCCAAGCTTGCTGCCAAGAACAAAGCGTCT-3'; 5'-GCCTCTAGATCCCGCAGCGCCGATT-3') were used for amplification of ~500 bp size amplicon of *pilT* gene homolog in F1C1. This amplicon was sequenced for confirming homology with the *pilT* as well as *rpoN2* sequences of GMI1000 strain. Amplicon was ligated to a T-A cloning

vector pTZ57R/T (Thermo Fisher Scientific) to get a construct pNST1 (pTZ57R/T::$pilT_{F1C1}$) following instructions of manufacturing company. pNST1was subjected to restriction digestion with *Hin*dIII and *Xba*I enzymes simultaneously. The resulting ~500bp amplicon from the previous step was gel-extracted and subsequently ligated to pCZ367 (Cunnac et al., 2004) vector that harbors a promoterless *lacZ* reporter gene and Ampicillin, Gentamycin selection markers. Prior to ligation set-up, pCZ367 vector was linearized with the same pair of restriction enzymes used in digestion of the amplicon. Recombinant plasmid pNST001 (pCZ367::$pilT_{F1C1}$) was isolated from transformed DH5α cells followed by confirmation of cloning step with digestion of pNST001 with *Hin*dIII and *Xba*I enzymes.

Primers (5'-GCCAAGCTTGCGACCGAATTTGCACAGG-3'; 5'-GCCTCTAGACGTCTTCGGCCTCGATCAT-3') were used to amplify ~1.3 kb amplicon of *rpoN2* homolog in F1C1. This amplicon was confirmed for homology with *rpoN2* gene in GMI1000 by nucleotide sequencing. This amplicon was first cloned into pTZ57R/T vector to obtain pNSN2 (pTZ57R/T::$rpoN2_{F1C1}$) and subsequently the same amplicon was ligated into pCZ367 vector to obtain vector construct pNSN2001 (pCZ367::$rpoN2_{F1C1}$). Cloning steps were same as discussed above.

The recombinant vector constructs pNST001 and pNSN2001 were naturally transformed into *R. solanacearum* F1C1 strain to create insertion mutations in *pilT* and *rpoN2* gene homologs of F1C1 respectively. Successful transformants were selected on BG-agar plates containing ampicillin and gentamycin antibiotics. Transformants were checked for positive X-gal activity. Insertion mutations in *pilT* and *rpoN2* genes of successful transformants were confirmed by PCR. Phenotype of *pilT* mutant was also studied for twitching motility deficiency (SFig 7c) following method of Liu et al., 2001. One insertion mutant from each of the above namely

TRS1014 (F1C1::pCZ367::*pilT*) and TRS1008 (F1C1::pCZ367::*rpoN2*) was recruited for subsequent experiments.

Creation of *hrpB and phcA* mutant of *R. solanacearum* F1C1. To create *hrpB* and *phcA* mutations in F1C1 background, genomic DNA samples from the *hrpB* mutant (GMI1525; Genin et al., 1992) and *phcA* mutant (GMI1605; Genin et al., 2005), both created in GMI1000 strain background, were used to naturally transform wild type F1C1 individually. Transformants for both types were selected on BG-agar plates supplemented with spectinomycin antibiotic. Two of the transformants from previous step, TRS1012 (*hrpB* mutant F1C1) and TRS1013 (*phcA* mutant F1C1) were recruited in subsequent studies. *hrpB* mutants of F1C1 were found deficient to elicit HR in tobacco leaves after infiltration (SFig 7a) as well as were found virulence deficient in tomato seedlings by the root inoculation unlike the wild type F1C1 (Fig 3). The *phcA* mutant colonies were transparent due to deficient for exopolysaccharide production, exhibited very high motility on semisolid agar medium (SFig 7b, and was virulence deficient unlike the wild type F1C1 (Fig 3).

RESULTS

***R. solanacearum* pathogenicity in tomato seedlings by a root inoculation method.** An initial observation in the author's laboratory regarding development of pathogenicity symptoms in 6-7 days old tomato seedlings under gnotobiotc root-inoculation of *R. solanacearum* confirmed susceptibility of early stages of tomato seedlings toward the pathogen (Kumar, 2014). In this set-up, each tomato seedling (S22 Evergreen variety) kept in a 1.5 microfuge tube containing 1.0 ml water was directly inoculated with *R. solanacearum* suspension. Subsequently this root inoculation method was found to be not effective against seedlings of other tomato cultivars tried in the laboratory. However, the seedlings of different tomato cultivars were susceptible to *R.*

solanacearum F1C1 infection by leaf clipping inoculation method developed in the authors' laboratory (Kumar et al 2017). Therefore, devising an efficient root inoculation method devoid of such constraints was imperative. While our efforts to find out an effective root inoculation method in 6-7 days old seedlings were continuing, in a serendipitous way we observed that a subtle change in inoculation approach significantly influenced *R. solanacearum* pathogenicity in tomato seedlings. *R. solanacearum* inoculated by the devised root inoculation method was proficient in causing pathogenicity in young tomato seedlings (Fig. 1a). Two steps in the inoculation process were found to be crucial (Fig 1b). Firstly, immersion of the tomato seedling root in *R. solanacearum* suspension followed by exposure of the root to air had tremendous influence on the pathogenicity symptoms developed in the seedlings. In our experiments the air exposure time has been kept as five minutes although an instant air exposure was also sufficient to result in virulence of the tomato seedlings. Secondly, direct addition of *R. solanacearum* suspension to root of tomato seedlings already submerged in water resulted in lesser or inconsistent disease symptom. In another way, i.e. directly transferring the seedlings into a microfuge already containing *R. solanacearum* suspension also did not result in very high number of wilting of the seedlings. Disease progression, as well as pathogenicity due to *R. solanacearum* was investigated in tomato seedlings after the root inoculation. It was notable to observe that the wilting symptoms as well as death of some inoculated seedlings appeared within 35 to 48 hours post inoculation. In fact, pathogenicity symptoms observed by the root inoculation method is faster than the leaf clipping inoculation method in which the disease symptom appeared on the 3rd days post inoculation (Kumar et al, 2017). On the 7th day post-inoculation via this root inoculation method, about 80-90% of the inoculated seedlings were found to be dead. To confirm that the death of the inoculated seedlings by this method was specific to *R.*

253 *solanacearum* inoculation, seedlings were also inoculated with few non-pathogenic bacteria such
254 as *Pseudomonas putida, Bacilus subtilis*, and *Escherichia coli*. After the 7th day post-
255 inoculation, none of the seedlings inoculated with these nonpathogenic bacteria exhibited disease
256 symptom (SFig 1; STable 1). This outcome indicated that death of the seedlings occurred due to
257 *R. solanacearum* F1C1 *via* this mode of inoculation. Further *R. solanacearum* F1C1
258 pathogenicity of similar magnitude could be observed in three other tomato cultivars [Durga
259 (Ruby), Akhilesh and Vijay] (SFig. 2; STable 2). We further studied F1C1 pathogenicity in
260 tomato seedlings by root inoculation as a function of the pathogen concentration in the inoculum.
261 Bacterial concentration varied from ~10^9 to ~10^3 $CFUml^{-1}$. Disease symptoms were observed
262 distinctly up to ~ 10^5 CFU/ml. It is also observed that the pathogenicity and disease progression
263 magnitude were decreasing as bacterial concentration in the inoculum decreased (SFig 3; STable
264 3).

265 The association of F1C1 with tomato seedling inoculated by this method was studied
266 further by using *R. solanacearum* F1C1 derived strain TRS1002 (*gus* marked) as well as
267 TRS1016 (mCherry marked) to study bacterial colonization in the seedlings. Bacterial
268 colonization in the infected seedlings was observed from root to the shoot regions (Fig.2a, Fig.
269 2b). This suggested that after the inoculation, the bacterium migrated from root to the shoot
270 regions during the infection process and resulted in pathogenesis.

271

272 **The root inoculation method can be used to study *R. solanacearum* virulence functions**. To
273 further evaluate that this root inoculation method is useful in studying virulence functions of *R.*
274 *solanacearum*, we inoculated tomato seedlings with different *R. solanacearum* mutants such as
275 *hrpB* (TRS1012), *phcA* (TRS1013), *rpoN2* (TRS1015) (Table 1). As anticipated, the *hrpB*

mutant was non-pathogenic, the *phcA* mutants was found to be significantly reduced for virulence, while the *rpoN2* mutant exhibited virulence proficiency (Fig. 3; S Fig/Table 4). The virulence phenotypes of *hrpB, phcA, rpoN2* mutants were in concordance with the virulence phenotype data reported earlier in grown up tomato plants (Ray et al 2015). A characteristic yellowish color of the cotyledon leaves was observed in case of seedlings inoculated with *phcA* mutant. A leaf clip inoculated *phcA* mutant however, had no such manifestations. Future investigation may reveal the reason behind this difference.

It has been reported in the literature that a twitching motility deficient strain (*pilT* mutant) of *R. solanacearum* is virulence deficient in tomato plants inoculated by soil drenching (Liu et al., 2001, Kang et al., 2002). We created a *pilT* mutant of F1C1 in this study (TRS1014), which was deficient for twitching motility (SFig 7c). TRS1014 was found to be moderately virulence deficient in tomato seedlings unlike the *hrpB* and *phcA* (Fig 4; S Fig/Table 5). This proved that root inoculation method in tomato seedling is useful to discriminate between severe virulent deficient strains and moderately virulent deficient strains of *R. solanacearum*. Interestingly, TRS1014 was further found to be virulence proficient by the leaf clip inoculation method in tomato seedlings (S Fig/Table 6). This might be due to differential requirement of twitching motility during the two mode of infection.

DISCUSSION

Here in this work we are documenting a root inoculation method to study *R. solanacearum* pathogenicity in early stages of tomato seedlings under gnotobiotic conditions. This method is effective for performing the pathogenicity assay in seedlings of different tomato cultivars. It was also found useful for studying the known virulence functions of *R. solanacearum* such as *hrpB*,

299 *phcA* and *pilT*. Through this inoculation process, *R. solanacearum* strains tagged with *gus* and
300 mCherry were recruited for observing bacterial colonization in tomato seedlings which
301 manifested colonization of the bacteria in root as well as shoot regions. The method is reasonably
302 simple, easy and rapid. As this involves less inoculum of bacteria as well as less space, large
303 scale screening of tomato seedlings for *R. solanacearum* virulence assay is possible by this
304 method. It is pertinent to note that previously root inoculation of this pathogen has been
305 performed in plantlets of *Medicago truncatula* as well as tomato by germinating seeds under
306 sterile conditions, in presence or absence of nutrients (Vasse et al., 1995; Vailleau et al., 2007).
307 Because of the involvement of tissue culture based techniques, these methods will not be easy for
308 doing pathogenicity study in large scale. Recently Lu et al (2017) have reported *R. solanacearum*
309 root infection in early stages of *Arabidopsis thaliana* under gnotobiotic condition.

310 The extensive pathogenicity of *R. solanacearum* in 6-7 days old tomato seedlings within
311 48 h of root inoculation is the demonstration of its virulence in very early stages of plant.
312 Though similar pathogenicity in tomato seedlings was reported earlier by the same authors using
313 leaf clipping inoculation, the pathogenicity in seedling by root inoculation holds much
314 significance than the leaf clipping inoculation because the pathogen enters the host plant through
315 its root during natural infection. An interesting point regard to leaf-clipping where disease
316 symptoms in tomato seedlings was noticed to begin at the point of inoculation then progressing
317 downward (Kumar et al., 2017), through this root inoculation process, disease appearance could
318 be observed in the shoot region without any visible sign of effect at the root region. Further, the
319 characteristic bending of the upper shoot region in the beginning of the disease in seedling was
320 observed by root inoculation, which was not observed in seedling by leaf clipping inoculation.
321 This indicates that infection in the tomato seedlings by the two modes of inoculation may not be

identical. This is supported by virulence deficiency of *pilT* mutant through the root inoculation method but not by leaf clipping method. A recent report claims that *R. solanacearum* has to overcome the host root cell fabricated nucleic acid network in order to successfully invade the root (Tran et al, 2016). It is likely that *R. solanacearum* entry mechanism through root might be complicated than by any shoot inoculation mechanism such as leaf-clipping. It is pertinent to note that previously *R. solanacearum* mutants deficient in swimming motility or in aerotaxis were reported to be virulence deficient through the root inoculation by soil drenching method whereas were virulence proficient inoculated by petiole cut method in tomato plants (Tans-Kersten et al, 2001, 2004;Yao and Allen, 2007).

The difference in the number of tomato seedlings dying as a function of bacterial concentration in the inoculum is interesting. It indicates that bacterial initial population during infection and their growth inside the seedlings are important for the disease symptom. How the pathogen population affects the disease in plants in case of *R. solanacearum*, which is a systemic pathogen in comparison to other bacterial pathogens that are tissue specific, is an interesting question for future research.

In the presented method, immediate exposure of root to air after instant dipping in the bacterial inoculums was found to be crucial for aggressive infection and disease progression in tomato seedlings. The importance of exposure to air was a serendipitous finding in our study. The air exposure time for root in our experiment was set for five minutes (Fig 1 a/b), although even an instantaneous exposure to air is sufficient to cause pathogenicity symptoms in tomato seedlings. While precise role of air exposure is unknown, however, claims of Yao & Allen (2007) regarding involvement of 'aerotaxis' in *R. solanacearum*-tomato plant interaction, might indicate involvement of an identical mechanism during the pathogen-tomato seedling

345 impingement. The authors (Yao & Allen, 2007) tested *R. solanacearum* root colonization by
346 incubating tomato seedlings in bacterial inoculums for 30 minutes, although there was no
347 mention regarding virulence in them. Probable role of 'air exposure' or 'aerotaxis' may be also
348 predicted in the root-dip assay conducted by Park et al. (2007) in six week old tomato plants
349 where they incubated tomato plant roots in bacterial suspension for equal duration as in case of
350 Yao & Allen (2007) before transferring the plants to soil. Similarly, Maji and Chakrabartty
351 (2014) in their pathogenicity assay immersed surgically wounded roots of tomato plantlets in *R.*
352 *solanacearum* suspension for 3 hours. Unknowingly, the transient period between transfers of the
353 plants from bacterial suspension to respective culture conditions in their experiments might have
354 played a role during infection progression. In addition to the above, during our infection study,
355 we observed an interesting infection behavior of *Ralstonia solanacearum* in case of pre-wet
356 tomato seedling. When tomato seedlings were incubated for 24 h in a microfuge tube with the
357 root region submerged in sterile water, and then inoculated with *R. solanacearum* by the root
358 inoculation method, the seedling were surprisingly found to be less susceptible to wilting
359 symptom. (STable/Fig. 8). However, bacterial colonization was observed inside the inoculated
360 pre-wet tomato seedling. It is interesting to note that the tomato seedling under the same pre-wet
361 condition, were susceptible to *R. solanacearum* pathogenicity when inoculated by leaf clipping
362 method. Whether *R. solanacearum* is deficient in its attachment to the roots of the pre-wet
363 tomato seedlings will require further investigation.

364 Considering the potential benefits of the root inoculation method described here, we
365 anticipate this inoculation method will help the world scientific community to address several
366 fundamental questions pertaining to *R. solanacearum* interaction with seedling stages of the host
367 and aid in foreseeing mechanism of virulence in adult plants. This method might turn out to be

368 instrumental in devising suitable biocontrol measures against the wilt pathogen in immediate
369 future.

370

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384

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Table 1: bacterial strains and plasmids used in this study

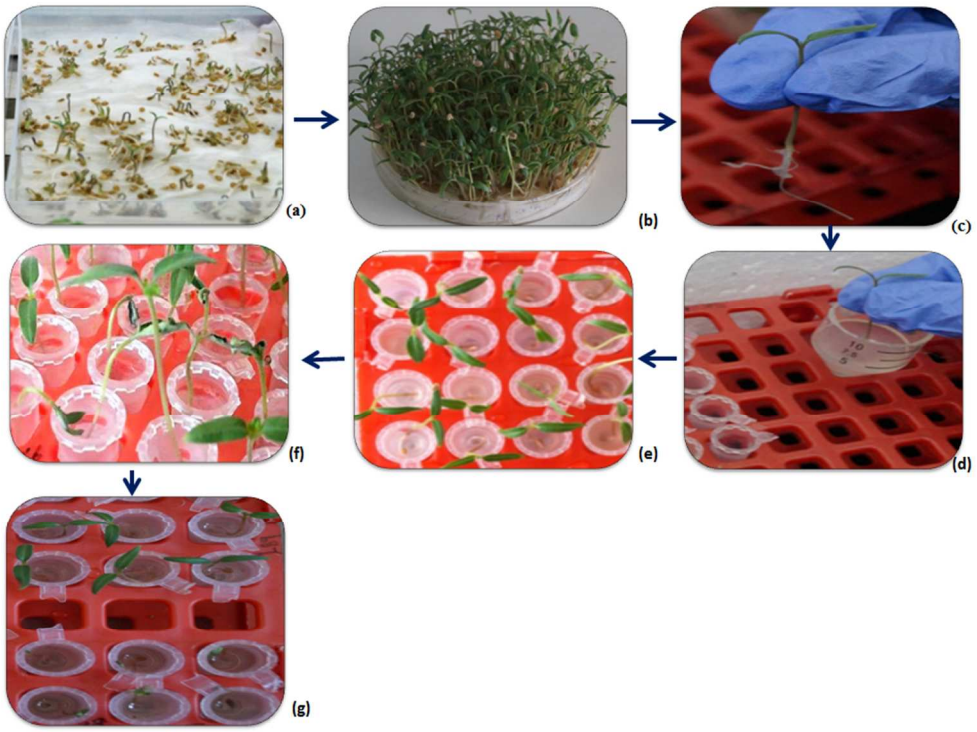
***Ralstonia solanacearum* strains**			
Sl no.	**Strain**	**Characteristics**	**Reference/Source**
1	F1C1	Wild type virulent *R. solanacearum* strain (Phylotype I). It was isolated from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India.	Kumar *et al*., 2013
2	TRS1001	*rif-1*; Rif^r, Vir^+ strain derived from F1C1, the strain was selected as a spontaneous Rif mutant from F1C1 culture.	Kumar, 2014
3	TRS1002	*rif-1 zxx::Tn5gusA11;* Gus +ve, Rif^r, Spc^r, Vir^+, this strain is derived from TRS1001 after *Tn5gusA11* insertion in an unknown locus in the genome.	Kumar, 2014
4	TRS1012	*hrpB*::Ω; Spc^r, HrpB deficient, Vir^-, Hypersensitive response deficient (HR^-), derived from F1C1	This work
5	TRS1013	*phcA*::Ω*;* Spc^r, PhcA deficient, exopolysaccharide deficient (EPS^-), hypermotile, derived from F1C1	This work

6	TRS1014	*pilT::pNST001*; Amp^r and Gen^r, PilT deficient, Vir^-, twitching motility deficient, derived from F1C1	This work
7	TRS1015	*rpoN2::pNSN2001*; Amp^r and Gen^r RpoN2 deficient, Vir^+, derived from F1C1	This work
8	TRS1016	Gen^r, mCherry tagged F1C1	This work

Escherichia coli **and other bacterial strains**

Sl no.	Strain	Characteristic	Reference/Source
1	DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A*sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Lab collection
2	N4T	*Pseudomonas putida*	Lab collection
3	C6a	*Bacillus subtilis*	Lab collection
		Plasmids	
1	pGEMT	Amp^r; Cloning vector	Promega
2	pTZ57R/T	Amp^r; Cloning vector	Thermo Scientific
3	pCZ367	Amp^r; Gen^r ; Insertional vector with *lacZ* reporter	Cunnac et al., 2004
4	pNST1	pTZ57R/T::*pilT*	This work

5	pNSN2	pTZ57R/T::*rpoN2*	This work
6	pNSN2001	pCZ367::*rpoN2*	This work
7	pNST001	pCZ367::*pilT*	This work



Pictures describing different steps involved the root inoculation method to study R. solanacearum pathogenicity in tomato seedlings

(a) Germination of tomato seeds on a sterile and wet tissue paper bed. Prior to spreading, seeds were washed and soaked for 24 h. Seeds were allowed to germinate in a growth chamber at 28 °C and 75% RH (relative humidity) up to the seedling stage.

(b) Germinated seedlings in a plate after 6-7 days.

(c) A 7-days old tomato seedling is taken out.

(d) Root inoculation of tomato seedling in pathogen inoculum by dipping the root.

(e) Each inoculated seedling transferred to sterile empty microfuge (1.5–2 ml) tubes. After ~5 minute exposer to air, 1.0 to 1.5 ml sterile water is added to the microfuge. The tray containing the microfuge tubes having the seedlings is kept inside the growth chamber.

(f) Within 48 h infected seedling started wilting.

(g) After 7 days 80%-90% of infected tomato seedlings were wilted/ died (lower side) in comparison to water control (upper side).

256x193mm (96 x 96 DPI)

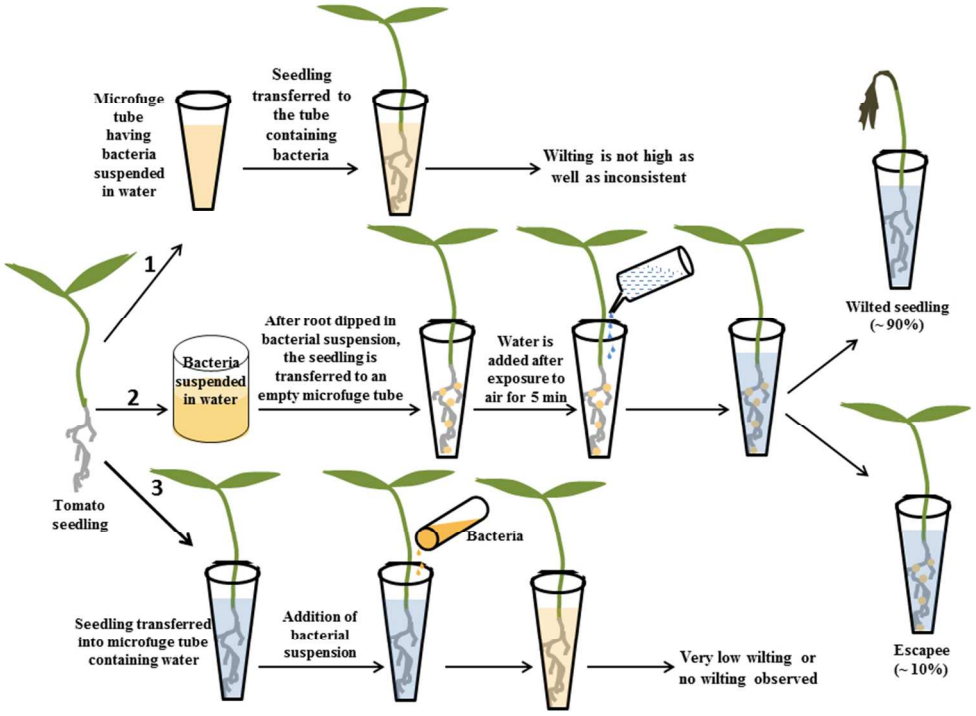


Fig. 1b┼ A schematic representation of the R. solanacearum pathogenicity in tomato seedlings by the root inoculation method┼ 6-7 days old tomato seedlings are root dipped in bacterial suspension and then transferred to microfuge tubes. After ~ 5 minutes expose to air, water is added to the microfuge and then incubated in the growth chamber. Wilting symptom started appearing from the 2nd days post inoculation onwards. To observe maximum wilting in the seedling the sequence number 2 is to be followed. In sequence 1 and 3 cases, infection found to be inconsistent and low. ┼

254x190mm (96 x 96 DPI)

Fig 2a: X-gluc staining of the seedlings root inoculated with R. solanacearum marked with gus ‼ ┼ Here in this picture one tomato seedling showing blue in color suggesting gus positive. For the gus assay this tomato seedling was root inoculated with a gus marked Ralstonia solanacearum strain (TRS1002). Blue color stain in root & shoot region confirming the bacterial colonization in pathogen infected seedling while other tomato seedlings are control seedling, where no bacteria have been inoculated appeared as gus negative. ‼ ┼

36x92mm (96 x 96 DPI)

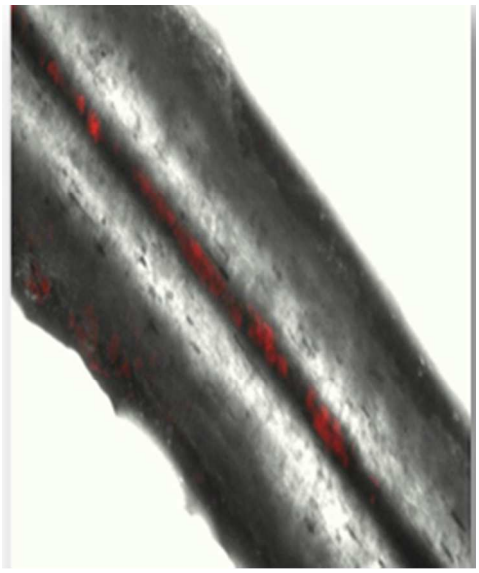
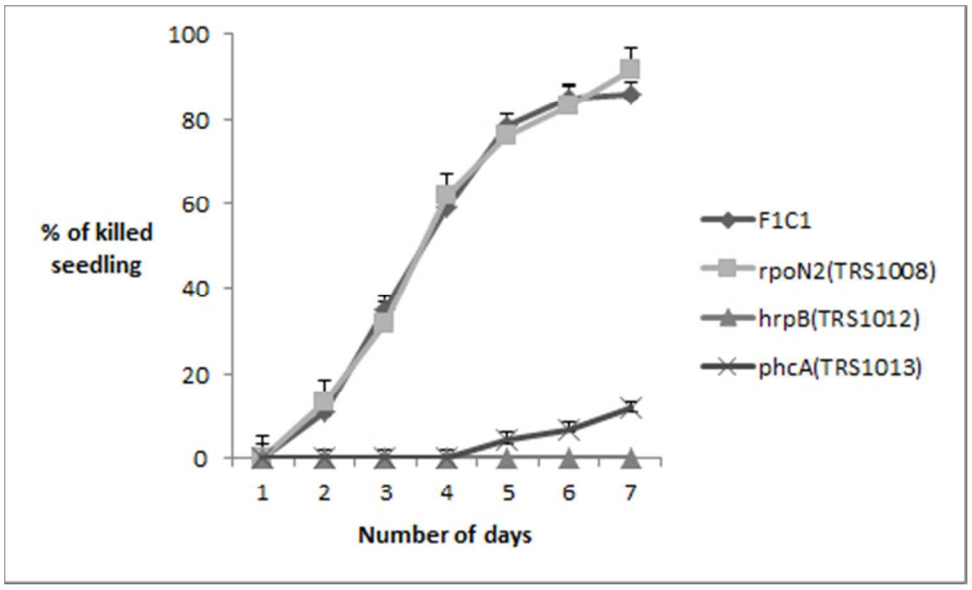


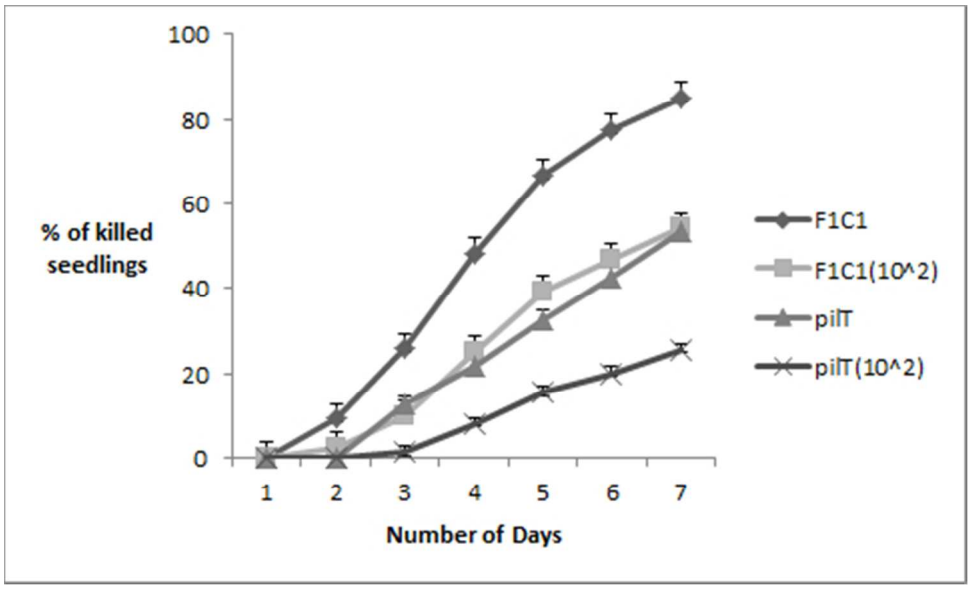
Figure 2b: Tracking the presence of R. solanacearum in tomato seedlings inoculated with mCherry labeled bacteria ┼ Further confirmation of pathogen colonization inside infected tomato seedling, we inoculated a m-Cherry tag R. solanacearum strain (TRS1016) in tomato seedling through root. Here in this image red fluorescence is observed in stem region, which is confirming the presence and colonization of m-cherry tag R. solanacearum inside tomato seedling. ┼

62x74mm (96 x 96 DPI)



Virulence data of F1C1, hrpB, phcA and rpoN2 strains in the root inoculated tomato seedlings x-axis represents the days post inoculation and y-axis represents the % of wilted seedlings. It is distinct that hrpB mutant is non-pathogenic while phcA mutant is significantly reduced for virulence, and rpoN2 strain is like the F1C1 wild type. The statistical significance has been shown in the S Fig 4.

127x76mm (96 x 96 DPI)



pilT is virulence deficient by root inoculation
x-axis represents the days post inoculation and y-axis represents the % of wilted seedlings. It is distinct that pilT mutant is reduced for virulence by root inoculation in comparison to the F1C1 wild type. We compared F1C1 wild type with pilT mutant in two different concentrations of bacteria in the inoculum (100 dilution and 102 fold dilution). In both concentrations pilT was found to be reduced for virulence. The statistical significance has been shown in the S Fig 5.

127x76mm (96 x 96 DPI)

Supplementary Tables and Figures

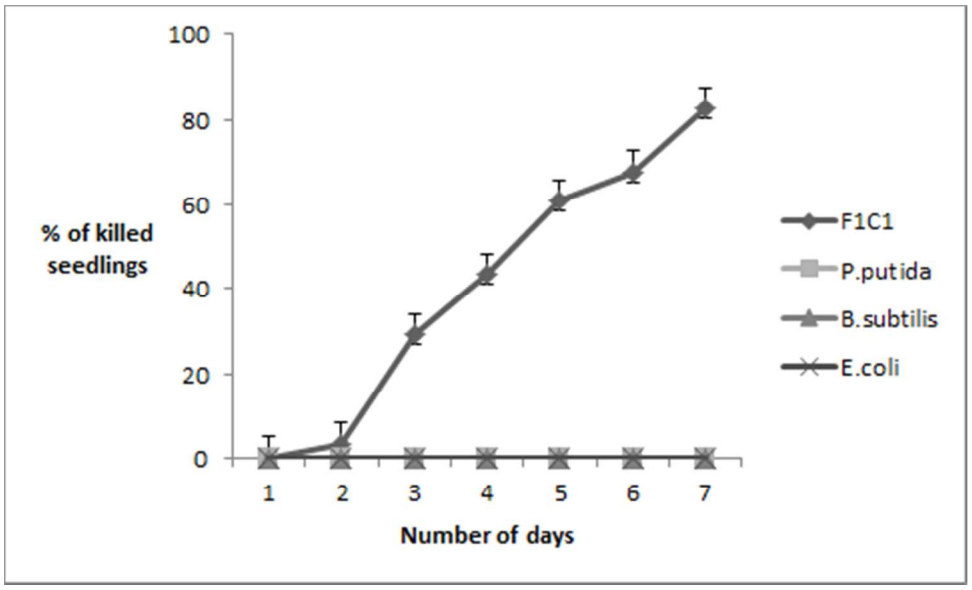
S Table 1

Virulence of non-pathogenic bacteria as well as F1C1 in tomato seedling by root inoculation

		F1C1	***P. putida***	***B. subtilis***	***E. coli***
Dpi 1	mean	0	0	0	0
	std.dev	0	0	0	0
Dpi 2	mean	1.3333	0	0	0
	std.dev	0.5774	0	0	0
Dpi 3	mean	11.6667	0	0	0
	std.dev	5.6862	0	0	0
Dpi 4	mean	17.3333	0	0	0
	std.dev	5.0332	0	0	0
Dpi 5	mean	24.3333	0	0	0
	std.dev	1.5275	0	0	0
Dpi 6	mean	27	0	0	0
	std.dev	1.732	0	0	0
Dpi 7	mean	33	0	0	0
	std.dev	1.732	0	0	0

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

S Fig 1



Virulence study of non-pathogenic bacteria in tomato seedlings by root inoculation

There was no disease in tomato seedling by non-pathogenic bacteria (*P. putida*, *B. subtilis*, *E. coli*) by the root inoculation method

S Table 2

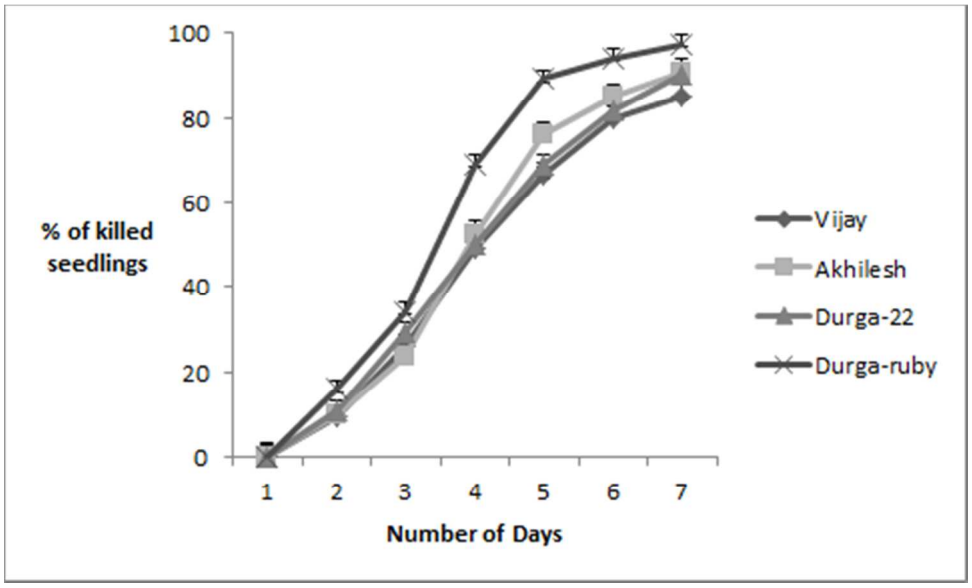
F1C1 wild type virulence in different tomato cultivars by the root inoculation method

		Vijay	Akhilesh	Durga-22	Durga-Ruby
Dpi 1	mean	0	0	0	0
	std.dev	0	0	0	0
Dpi 2	mean	3.6667	4	4.3333	6.3333
	std.dev	0.5774	1	0.5774	0.5774
Dpi 3	mean	10.3333	9.3333	11.6667	13.6667
	std.dev	1.1547	1.5275	1.1547	1.1547
Dpi 4	mean	19.6667	21	20	27.6667
	std.dev	1.5275	1	1	0.5774
Dpi 5	mean	26.6667	30.3333	27.6667	35.6667

	std.dev	1.5275	2.0817	1.5275	1.5275
Dpi 6	mean	32	34	32.6667	37.6667
	std.dev	2	1	0.5774	1.1547
Dpi 7	mean	34	36.3333	36	39
	std.dev	1	0.5774	1	1

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

S Fig 2



Wild type F1C1 pathogenicity in different tomato cultivars by root inoculation method

In the line diagram, it is showing the pathogenicity of F1C1 in four different tomato cultivars by the root inoculation method. x-axis represents the days post inoculation and y-axis represents the number of wilted seedlings. In this experiment virulence and disease progression was observed are very much similar in all four tomato cultivars.

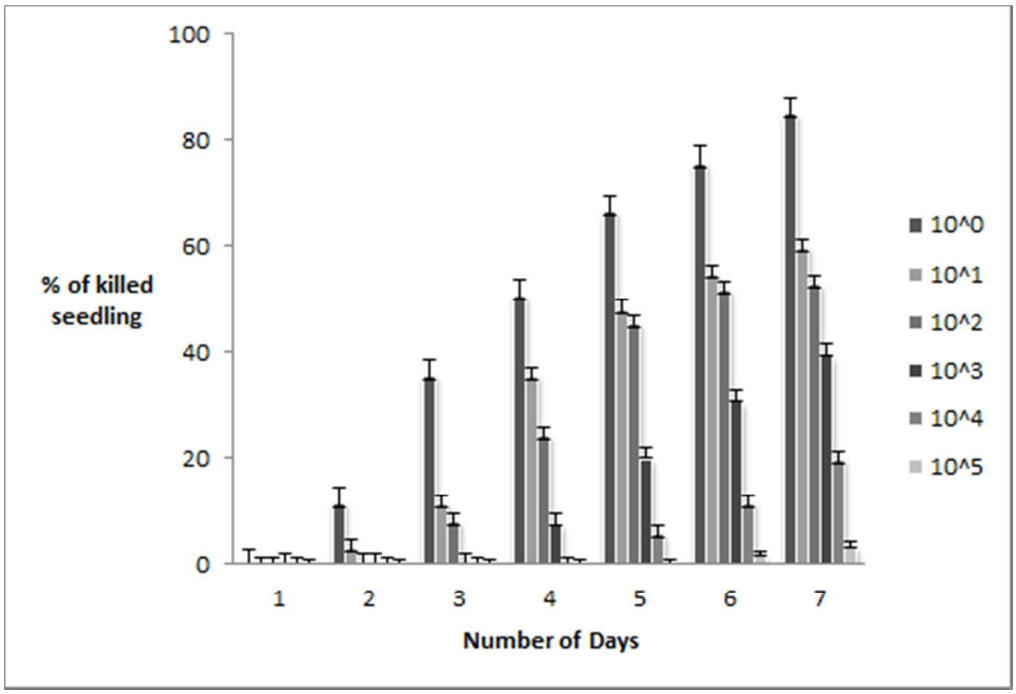
S Table 3

F1C1 virulence in tomato seedling as function of different concentration in the inoculum

DPI		Different dilution					
		10^0	10^1	10^2	10^3	10^4	10^5
Dpi 1	mean	0	0	0	0	0	0
	std.dev	0	0	0	0	0	0
Dpi 2	mean	4.6667	1.3333	0.25	0	0	0
	std.dev	0.5774	0.5774	0.5774	0	0	0
Dpi 3	mean	14.3333	4.6667	3.25	0	0	0
	std.dev	1.5275	0.5774	0.5774	0	0	0
Dpi 4	mean	20.3333	14.3333	9.75	3	0	0
	std.dev	1.1547	0.5774	1	1	0	0
Dpi 5	mean	26.6667	19.3333	18.25	8	2.3333	0
	std.dev	1.1547	0.5774	1	1	0.5774	0
Dpi 6	mean	30.3333	22	20.75	12.25	4.6667	0.6667
	std.dev	2.0817	1	0.5774	1.5275	0.5774	0.5774
Dpi 7	mean	34	24	21.25	15.75	8	1.3333
	std.dev	1	1	0.5774	1	1	0.3337

Wilting was observed distinctly up to 10^4 dilution (10^5 $CFUml^{-1}$). Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

S Fig 3



F1C1 virulence in tomato seedling as function of different concentration in the inoculum

In the line diagram, 1 to 7 showing bacterial inoculum concentration from 10^9 to 10^3 cfu/ml, x-axis represents the days post inoculation and y-axis represents the number of wilted seedlings. In this graph it is clear that by decreasing the bacterial concentration in the inoculum, virulence and disease progression of pathogen is also decreasing. Disease symptom and wilting in infected seedling was observed only up to 10^5 cfu/ml, while below 10^5 cfu/ml bacterial inoculum infected tomato seedling mostly behave like a water control tomato seedling and no any disease symptom appeared.

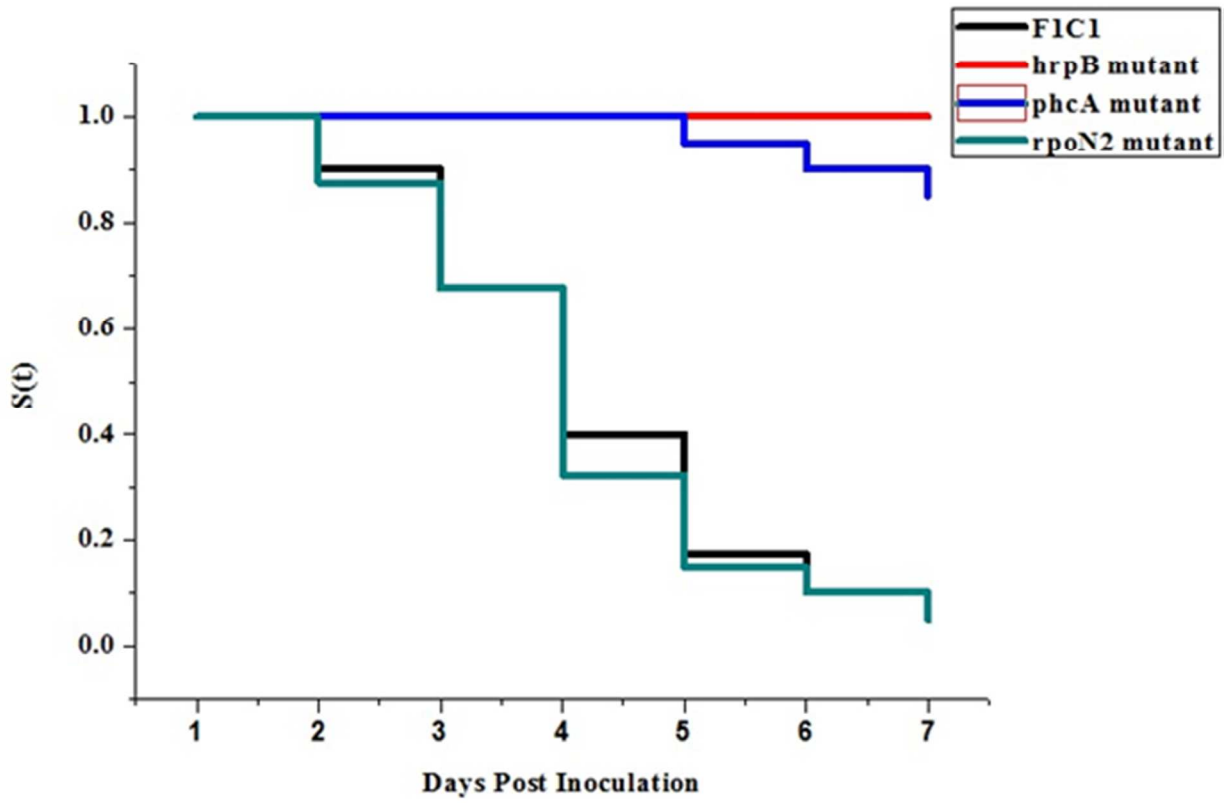
S Table 4

Virulence data of F1C1, hrpB, phcA and rpoN2 strains in the root inoculated tomato seedlings

		F1C1	phcA	hrpB	rpoN2	Control (H_2O)
Dpi 1	**mean**	0	0	0	0	0
	std.dev	0	0	0	0	0
Dpi 2	**mean**	4.3333	0	0	5.3333	0
	std.dev	0.5774	0	0	0.5774	0
Dpi 3	**mean**	14	0	0	12.6667	0
	std.dev	1	0	0	1.5275	0
Dpi 4	**mean**	23.6667	0	0	24.6667	0
	std.dev	0.5774	0	0	2.0817	0
Dpi 5	**mean**	31.3333	1.6667	0	30.3333	0
	std.dev	1.5275	0.5774	0	3.2146	0
Dpi 6	**mean**	34	2.6667	0	33.3333	0
	std.dev	2	1.1547	0	2.5166	0
Dpi 7	**mean**	34.3333	4.6667	0	36.6667	0
	std.dev	2.0817	1.1547	0	1.5275	0

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

S Fig 4



Kaplan–Meier survival probability[S(t)] curve for pathogenicity assay performed on tomato seedlings inoculated through root with wild type *Ralstonia solanacearum* (F1C1) and its three derivative mutants *phcA, hrpB and rpoN2*.

Here line Curves are clearly indicating the significant reduction of virulence behavior of *phcA,* and *hrpB* mutants, while *rpoN2* mutants showing no effect on virulence and disease progression in comparison to wild type ,so all these known mutants are showing similar behavior as reported earlier.

phcA, hrpB mutants were found to be significantly reduced in virulence as compared to the wild type F1C1($P < 0.05$; log-rank test) while in case of *rpoN2* mutant it shown similar virulence as wild type (F1C1)

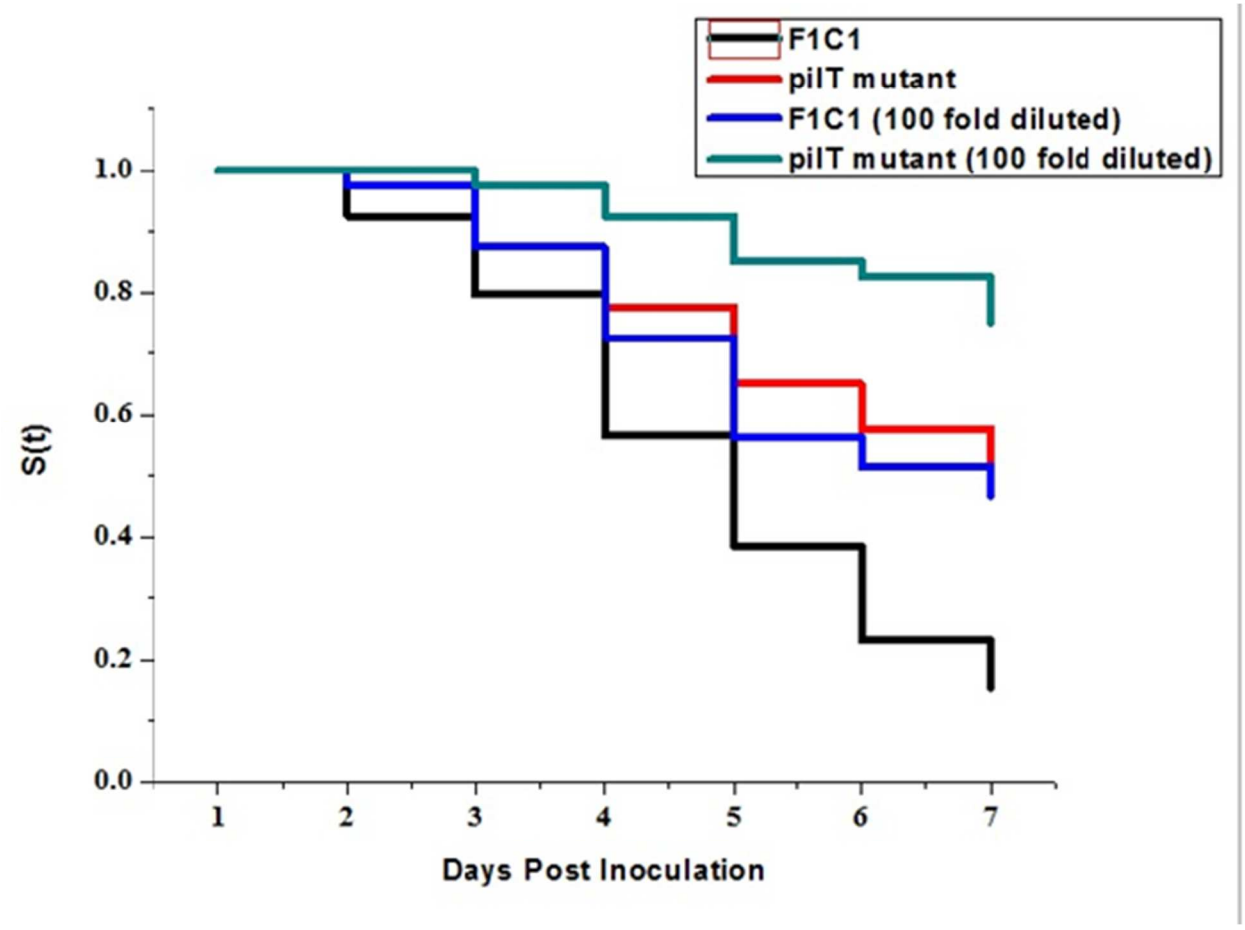
S Table 5

Virulence deficiency of *pilT* mutant by root inoculation

		F1C1	F1C1(10^2)	pilT	pilT(10^2)	Control
Dpi 1	mean	0	0	0	0	0
	std.dev	0	0	0	0	0
Dpi 2	mean	3.6667	1	0	0	0
	std.dev	0.5774	1	0	0	0
Dpi 3	mean	10.3333	4	5	0.5	0
	std.dev	2.0817	1	1	0.5774	0
Dpi 4	mean	19.3333	10	8.6667	3.25	0
	std.dev	2.0817	1	0.5774	0.5774	0
Dpi 5	mean	26.6667	15.6667	13	6.25	0
	std.dev	2.5166	2.5166	1	0.5774	0
Dpi 6	mean	31	18.6667	17	8	0
	std.dev	1	1.5275	1	1	0
Dpi 7	mean	34	21.6667	21.3333	10.25	0
	std.dev	1	1.5275	1.5275	0.5774	0

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

S Fig 5



Kaplan–Meier survival probability[S(t)] curve for pathogenicity assay wild type *Ralstonia solanacearum* (F1C1) and its derivative *pilT* mutant strain in tomato seedlings through root inoculation

During infection assay two different pathogen inoculum concentration were used one is saturated while other is 100 fold diluted, in this figure line graph is clearly showing significant reduction

in virulence of *pilT* mutant in comparison to wild type *Ralstonia solanacearum*. Which is found in in both case of inoculum, saturated as well as 100 fold dilution condition, statically it also found to be significantly deficient in virulence compared to the wild type F1C1($P < 0.05$; log-rank test)

S Table 6

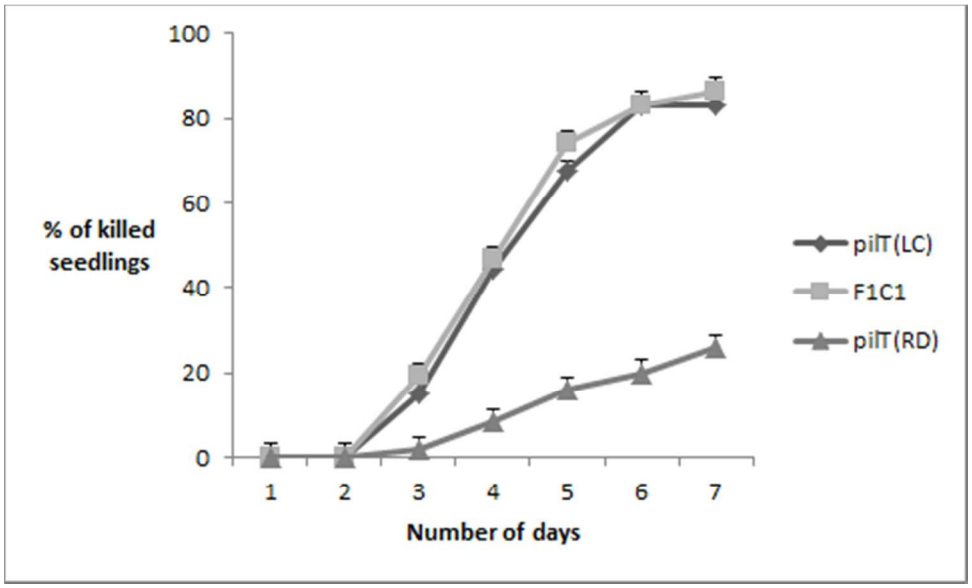
pilT is virulence proficient by leaf clipping

		F1C1(LC)	pilT(LC)	pilT(RD)	Control
Dpi 1	mean	0	0	0	0
	std.dev	0	0	0	0
Dpi 2	mean	0	0	0	0
	std.dev	0	0	0	0
Dpi 3	mean	7.6667	6	0.6667	0
	std.dev	1.5275	1	0.5774	0
Dpi 4	mean	18.6667	17.6667	3.3333	0
	std.dev	1.5275	1.5275	0.5774	0
Dpi 5	mean	29.6667	27	6.3333	0
	std.dev	1.5275	1	0.5774	0
Dpi 6	mean	33.3333	33.3333	8	0
	std.dev	1.5275	2.0817	1	0
Dpi 7	mean	34.6667	33.3333	10.3333	0
	std.dev	1.5275	2.0817	0.5774	0

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

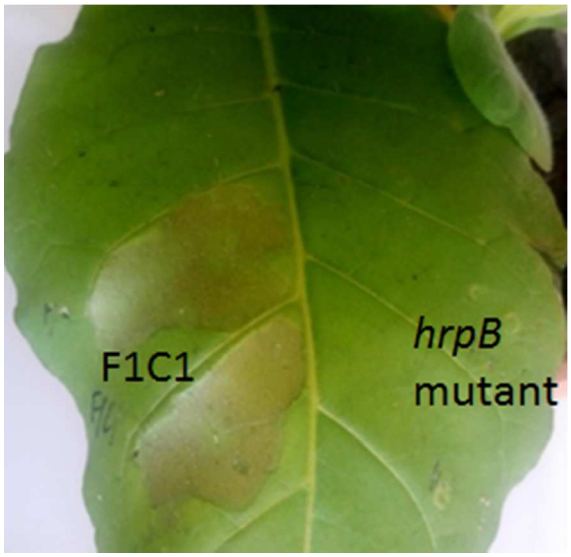
S Fig 6

pilT is virulence proficient by leaf clipping



x-axis represents days post inoculation and y-axis represents the mean of the number of seedlings wilted. The wild type F1C1 and the *pilT* mutant exhibit virulence of similar magnitude by leaf clipping whereas the same *pilT* mutant is reduced for virulence when root inoculation in tomato seedling. It is pertinent to note that F1C1 virulence by leaf clipping and root inoculation is similar in magnitude (data not shown). Mean and standard deviation is of three independent experiments.

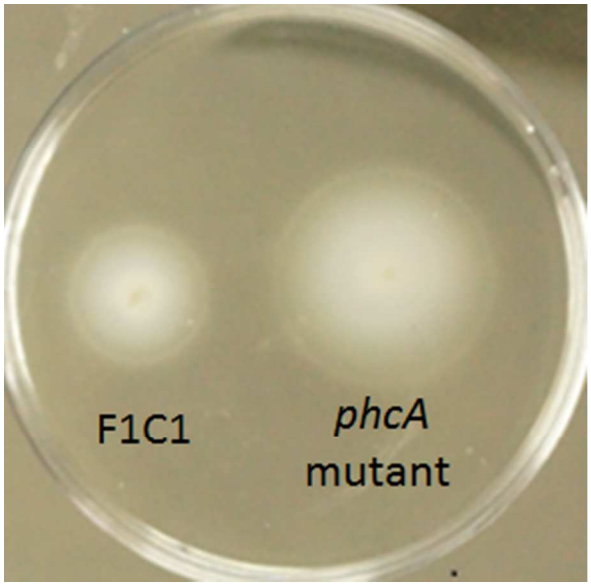
S Fig 7a



***hrpB* mutant (TRS1012) is deficient for eliciting HR in tobacco leaves**

Hypersensitive response of wild type (F1C1) as well as it derivative mutant *hrpB* in tobacco plant leaf. It is clearly showing that *hrpB* mutant(right side in leaf) is not eliciting hypersentive response (HR), while in case of F1C1 (wild type) (in left side of leaf) is eliciting HR in tobacco leaf.

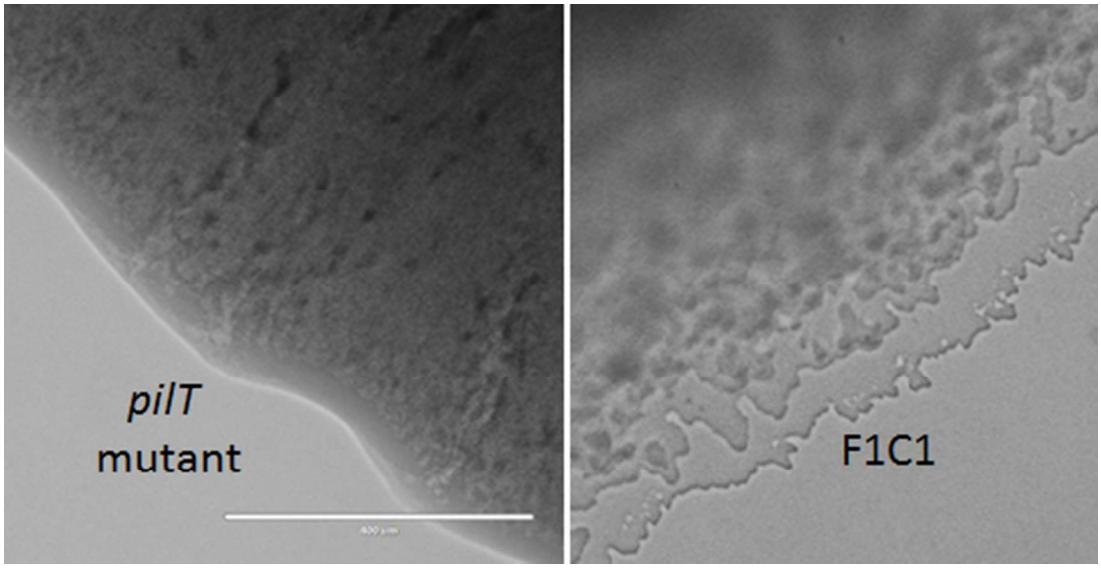
S Fig 7b



***phcA* mutant (TRS1013) is hypermotile**

Here in this picture showing the two whitish circular spots, in which larger circle in the right side is of the *phcA* mutant while smaller one in left side is by wild type (F1C1) bacterial strain. The motility test has been done in the soft agar (0.2 %) BG medium (Ray et al, 2015). *phcA* mutant is known to be hypermotile. In addition these mutants were found to be deficient for exopolysaccharide as well as virulence.

S Fig 7c



***pilT* mutant (TRS1014) is deficient for twitching motility**

Here in this picture it showing the Ralstonia solanacearum colony edge of wild type (F1C1) (right side) as well as its *pilT* mutant (left side) grown on BG medium. Edge shape are distinctly different in mutant one (left side) in comparison to wild type F1C1. *Ralstonia solanacearum*, smooth edge of mutant clearly indicating the absence of twitching motility while in case of wild type edge are have tiny projection like structure because of presence of twitching motility.

S Table 7

Primers used in this study

Sl No	NAME	PRIMER **Sequence (5'->3')**	reference
1	ONSM007	GCCAAGCTTGCGACCGAATTTGCACAGG	Forward

			(rpoN2)
2	ONSM008	GCCTCTAGACGTCTTCGGCCTCGATCAT	Reverse (rpoN2)
3	ONSM005	GCCAAGCTTCTCTCGAAATGCGTGCGAAG	*rpoN2* upstream to confirm the pNSN2001 insertion in TRS1015
4	OlacZR1	AAGGGGGATGTGCTGCAAGG	*lacZ* primer used to confirm the mutation in TRS1015
5	ONSPT01	GCCAAGCTTGCTGCCAAGAACAAAGCGTCT	Forward (pilT)
6	ONSPT02	GCCTCTAGATCCCGCAGCGCCGATT	Reverse (pilT)

S Table 8

***R. solanacearum* pathogenicity in normal tomato seedlings and prewet tomato seedlings**

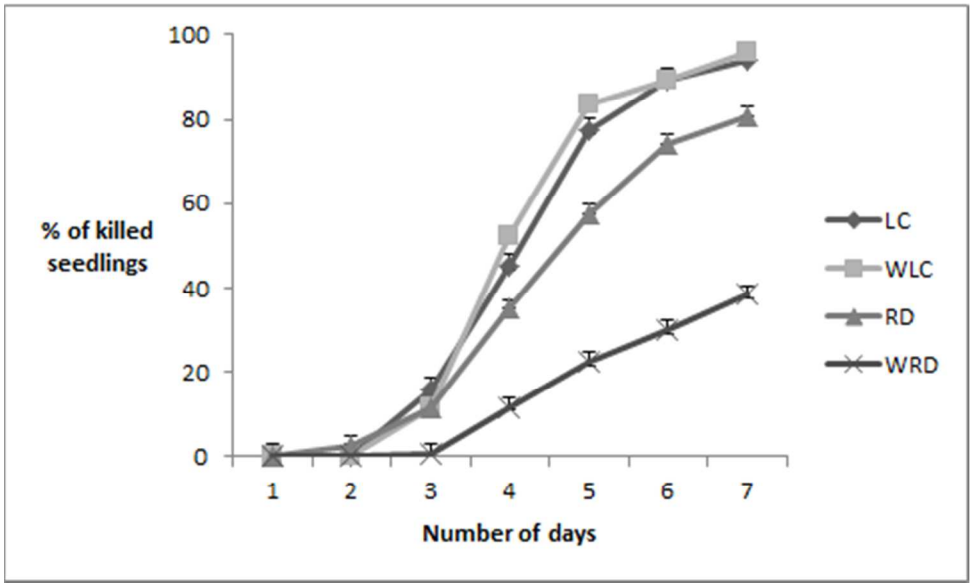
		LC	WLC	RD	WRD
Dpi 1	**mean**	0	0	0	0
	std.dev	0	0	0	0
Dpi 2	**mean**	0	0	1	0
	std.dev	0	0	1	0
Dpi 3	**mean**	6.3333	4.6667	4.6667	0.3333
	std.dev	0.5774	1.5275	0.5774	0.5774
Dpi 4	**mean**	18	21	14	4.6667
	std.dev	2	1	1	1.5275
Dpi 5	**mean**	31	33.3333	23	9
	std.dev	1	1.5275	1	2.6458
Dpi 6	**mean**	35.6667	35.6667	29.6667	12
	std.dev	0.5774	1.1547	1.5275	1.7321

Dpi 7	**mean**	37.6667	38.3333	32.3333	15.3333
	std.dev	0.5774	0.5774	0.5774	2.5166

Mean and standard deviation is of three independent experiments. In each inoculation in a set 40 seedlings were recruited.

RD: root inoculation; WRD: prewet root inoculation; LC: leaf clip inoculation; WLC: prewet leaf clip inoculation

S Fig 8



R. solancaerum virulence is reduced in prewet tomato seedling by root inocuation

In this graph x-axis represents days post inoculation and y-axis represents % of seedlings killed after inoculation. From the line grapgh it is observed that disease in case of prewet seedlings is reduced in comparison to the normal seedlings when inoculated by root. But in case of leaf clip inoculation disease is similar in both the normal and the prewet seedlings.