Appendix I

Characterization of RSc1775 gene homologue in *R. solanacearum* F1C1

1. Introduction:

Surface localized or secreted proteins called as adhesins are important for bacterial adherence to host tissue surfaces. The *R. solanacearum* genome is characterized by an unusually high number of adhesion genes, however very little information is known about adhesion functions of this bacterium. These adhesion functions are mainly secreted via type V secretion mechanism (Henderson *et al*, 2004**).** It may be classical autotransporter mode (known as Va) or classical two-partner secretion mode (known as Vb) or trimeric autotransporter type (known as Vc).

In *R. solanacearum* GMI1000 strain, 27 hemagglutinin adhesion genes are present both in chromosome and megaplasmid (Salanoubat *et al*., 2002). Among them, 13 gene products are called probable hemagglutinin and remaining 14 are called hemagglutinin like proteins. The large hemagglutinin polypeptides after their synthesis are translocated to cytoplasmic surface using sec-protein apparatus. After that they are translocated to outer membrane by using two-partner secretion system.

Here, we are interested to characterize *RSc1775,* one of the filamentous hemagglutinin homologue by creating insertion mutation in F1C1 strain of *R. solanacearum.*

2. Objectives:

i. Creation of *RSc1775* insertion mutant of *R. solanacearum* F1C1

ii. Expression study in BG and Minimal Medium

iii. Virulence study in tomato seedlings

3. Results

3.1 Creation of *RSc1775* insertion mutant of *R. solanacearum* F1C1

We used *lacZ* reporter gene fusion approach to create the insertion mutagenesis in RSc1775 homologue. We took reference sequences of GMI1000 to design the primers for its partial amplification in F1C1 strain. Forward primer,

oTP007 (5'- GCCAAGCTTCCACCACGTCCTTCGACAACA-3') incorporated with *HindIII* restriction site and reverse primer oTP008 (5'- GCCTCTAGAGGGAAAC TGAGCGGTGCG -3') incorporated with *XbaI* restriction site at their 5' ends respectively, were used to amplify ~ 1 kb amplicon of the gene. Amplification was performed in a PCR reaction volume consisting of 2.0 µl of 10X Dream Taq buffer, 1.2 µl of 2 mM dNTP mix, 0.6 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 to 20 µl 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 95°C for 1 min; annealing at 57°C for 30 sec; extension at 72°C for 30 sec and (step3) final extension at 72°C for 10 min. Amplified fragment was cloned into pTZ57R/T vector and confirmed by sequencing. Subsequently, recombinant construct was cloned into pCZ367, a promoter less insertional vector and finally recombinant *RSc1775::pCZ367* construct was transformed into F1C1 strain. Transformants were confirmed by PCR amplifications with primers oTP025 (5'-GCCCTGACGATCCACGACGACAC-3'); designed upstream of the *RSc1775* cloned fragment and olacR1 (5'-AAGGGGGATGTGCTGCAAGG-3'); designed downstream of *lacZ* gene and was recruited (Fig.1). Among many transformants we recruited TRS1028 strain for further studies.

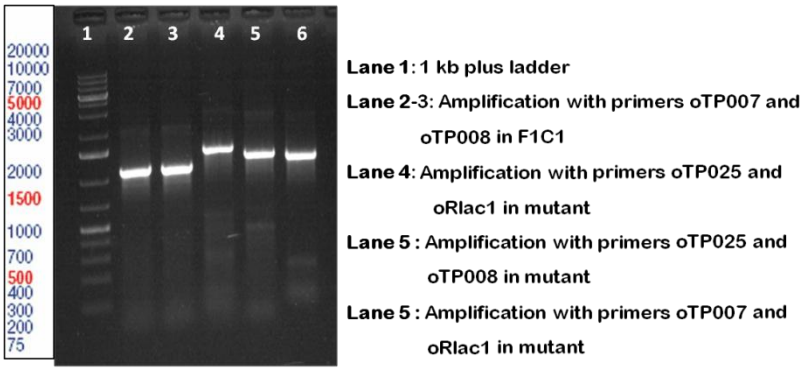


Fig.1: Agarose gel showing confirmation of insertion mutation in *RSc1775* of F1C1. Lane1: 1 kb plus DNA ladder. Lane 2 and 3: PCR amplification of partial fragment of *RSc1775* with primer pair oTP007 and oTP008 from wild type F1C1 and mutant respectively yielded ~1000 bp size DNA band. Lane 4: PCR amplification with primer pair oTP025 and oRlac1 in the mutant yielded ~1500 bp size DNA band. Lane 5: PCR amplification with primer pair oTP008 and oTP025 in the mutant yielded ~1200 bp size DNA band. Lane 6: PCR amplification with primer pair oTP007 and oRlac1 in the mutant yielded ~1200 bp size DNA band. The size of different DNA bands is showing at the side of the gel.

This sequence of the RSc1775 homologue were subjected to homology search (BlastN, NCBI) that showed its significant homology to corresponding gene in already sequenced genomes of different strains.

3.2 *RSc1775* homologue showed higher expression in Minimal Medium

We checked the expression of RSc1775 homologue in mutant background i.e. in TRS1028 strain by assessing β-galactosidase activity of *lac*Z genes expressed under its own promoter. We have used nutrient rich BG medium and nutrient poor Minimal Medium and Soil extract (SE) (Barman, PhD thesis) conditions for the study. We observed highest expression of *RSc1775* in Soil extract medium followed by Minimal Medium in comparison to BG medium (Fig.2).

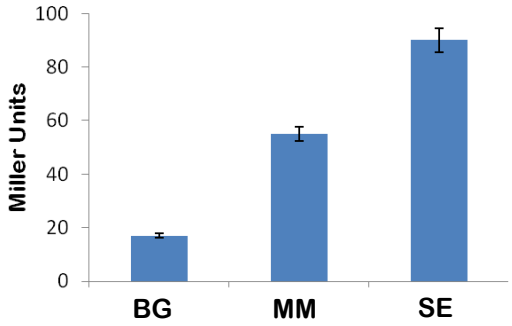


Fig.2: Expression analysis of the gene *RSc1775* in BG, MM and SE media by ONPG Assay

We have also checked its expression by quantitative RT-PCR in wild type F1C1 and found to be differentially expressed in Minimal media (Fig.3). The gene expression was analyzed with respect to the constitutively expressing *udp* gene. The threshold cycles (C_T) were averaged for triplicate reactions. The fold change value was calculated using ΔΔCT method.

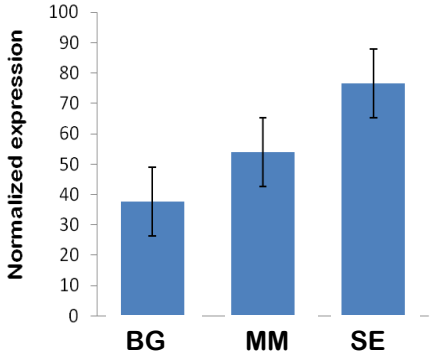


Fig.3: Expression analysis of the gene *RSc1775* in BG, MM and SE media by qRT-PCR.

R. solanacearum, the devastating vascular pathogen can thrives in nutrient poor plant xylem and expresses many pathogenicity genes during the wilt disease. Since minimal media mimic the *in planta* nutrient poor environment and this gene showed higher expression in these media in comparison with BG rich media, we expected that *RSc1775* gene might play a role during the pathogenesis or more specifically in initial adhesion of the pathogen to the host.

3.3 RSc1775 mutant showed reduced virulence in tomato seedlings

To check the role of *RSc1775* gene in virulence of *R. solanacearum* F1C1 if any, we performed virulence assay on tomato seedling host (30 seedlings in a set). Virulence assay was performed employing 6-7 days old tomato seedlings by root inoculation method (Singh et al., 2018). Moreover, we checked its virulence phenotypes by leaf clip inoculation method (Kumar et al. 2017) also (data not shown). Our virulence studies revealed that RSc1775 mutant was highly reduced for virulence by both root inoculation as well as by leaf clip inoculation method in compared to wild type F1C1. The result has been shown in the Fig.4.

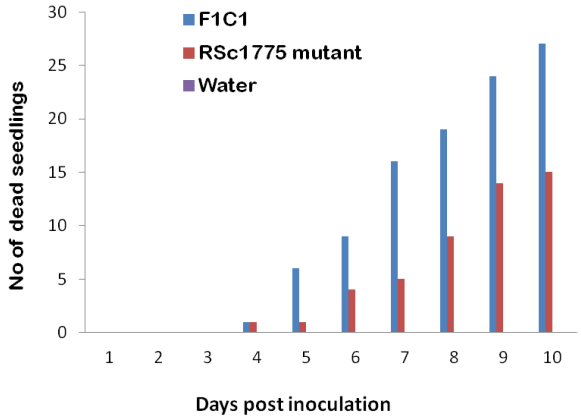


Fig.4: Virulence data analysis of *RSc1775* mutant by root inoculation. TRS1028 strain was inoculated in tomato seedlings by root dip method. Column graph showing number of seedlings killed against days post inoculation (DPI).

The virulence difference between F1C1 and RSc1775 mutant is statistically significant ($P < 0.001$; log rank test) as shown in Fig.5.

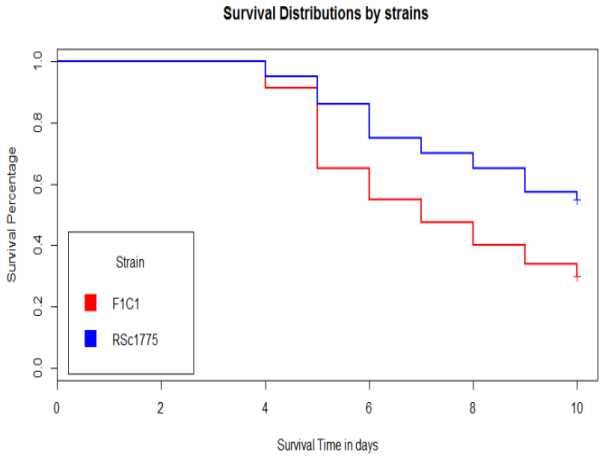


Fig.5: Kaplan–Meier survival curve depicting survival probability of infected tomato seedlings inoculated by root inoculation method. The *RSc1775* mutant exhibited significant virulence deficiency in compared to the wild type F1C1 ($P < 0.001$; log-rank test).

Appendix II

1. Media Composition

(i) BG	Concentration (g/l)
Peptone	10.0
Casamino acid	1.0
Yeast extract	1.0
Agar	15.0
Glucose	5.0
TZC	0.05

(ii) Minimal Media	Concentration (g/l)
$FeSO_4.7H_2O$	1.25×10^{-4}
$(NH_4)_2SO_4.7H_2O$	0.5
$MgSO_4.7H_2O$	0.05
KH_2PO_4	3.4

pH 7.0 is adjusted with KOH.

(iii) LB	Concentration (g/l)
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
NaCl	5.0

2. Reagents, Buffers and Solutions

(A) Buffers

(i) 50X TAE; electrophoresis buffer	Concentration (g/l)
Tris base	242.0 gm
0.5 M EDTA	100.0 ml
Glacial acetic acid	57.1 ml

(ii) Phosphate buffer	Concentration (M)
$Na_2HPO_4.7H_2O$	0.06

$NaH_2PO_4.H_2O$	0.04

pH was adjusted to 7.0.

(iii) Z- buffer	Concentration (M)
$Na_2HPO_4.7H_2O$	0.06
$NaH_2PO_4.H_2O$	0.04
KCl	0.01
$MgSO_4$	0.001
β-mercaptoethanol	0.05

Final volume was made up to 50.0 ml and p^H was adjusted to 7.0. Buffer was stored in 4˚C.

(iv) PBS buffer (1X)	Concentration (g/l)
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

(B) Reagents	Concentration (mg/ml)
(i) Ethidium bromide	10.0
(ii) IPTG	100.0
(iii) X- gal	50.0 (in DMF)
(iv) X- gluc	52.18 (in DMF)
(v) ONPG	4.0 (in 0.1 M Phosphate buffer; pH 7.0)
(vi) Congo-red	Concentration (% w/v)
Congo-red	0.1
(vii) Antibiotics (Stock)	Concentration (mg/ml)
Ampicillin	50.0 (in water)
Gentamycin	50.0 (in water)
Rifampicin	50.0 (in DMF)
Spectinomycin	50.0 (in water)

Nalidixic acid	50.0 (in water)
Kanamycin	50.0 (in water)

(C) Solutions for plasmid DNA isolation

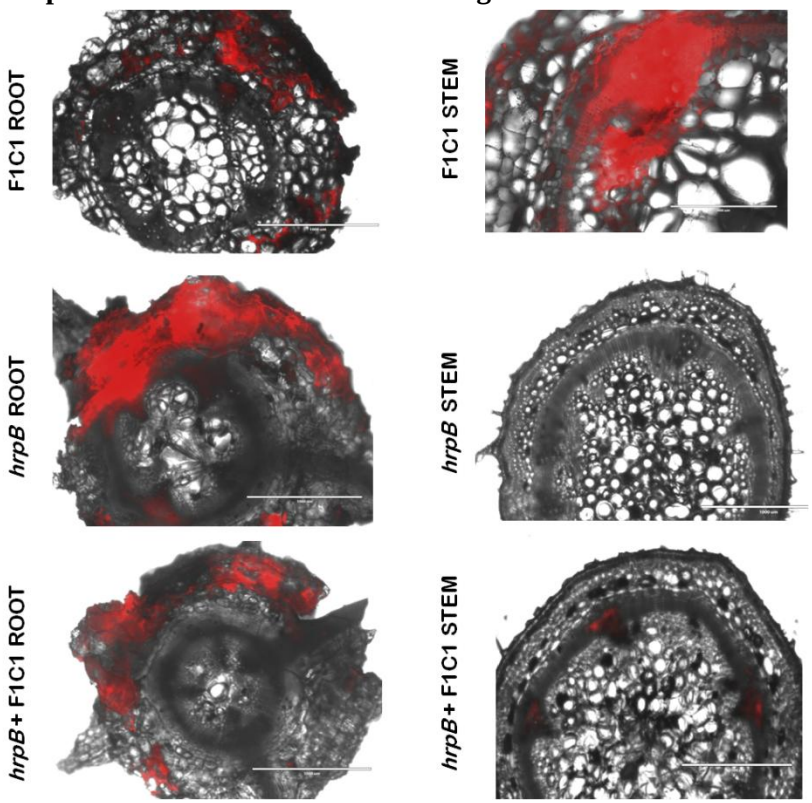
(i) Solution I	Concentration (gm %)
50 mM glucose	0.90
25 mM Tris-Cl (pH 8.0)	0.30
10 mM EDTA (pH 8.0)	0.37

(ii) Solution II	Concentration (gm %)
0.2 N NaOH	0.8
SDS	1.0

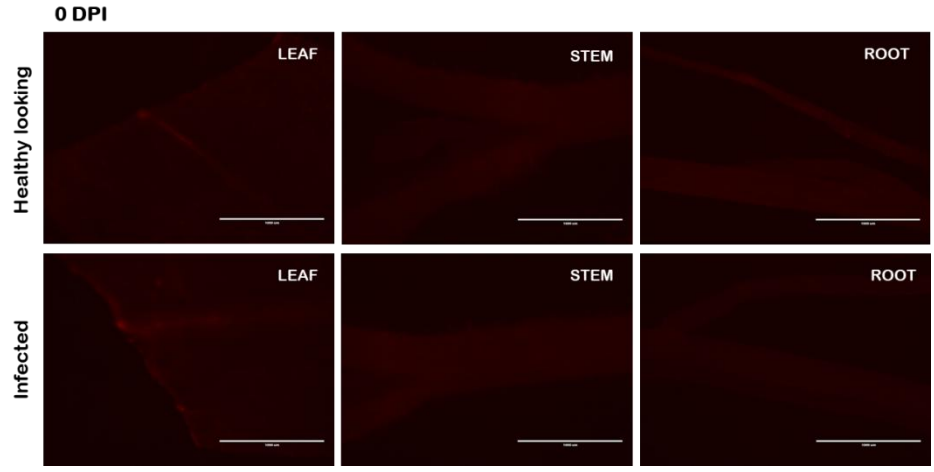
(iii) Solution III	Volume (ml)
5.0 M Potassium acetate	60.0
Glacial acetic acid	11.5
Autoclaved water	28.5

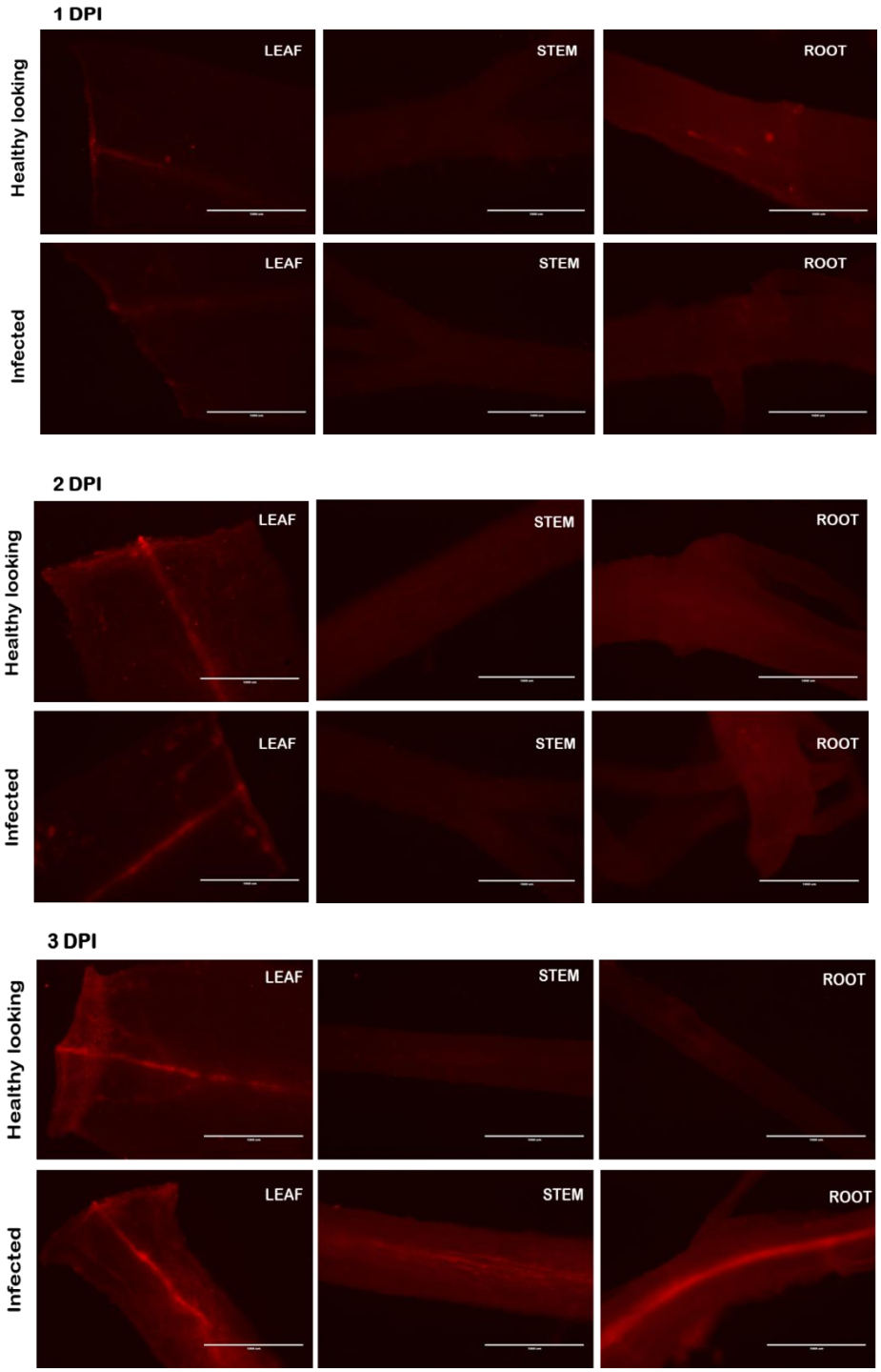
Appendix III

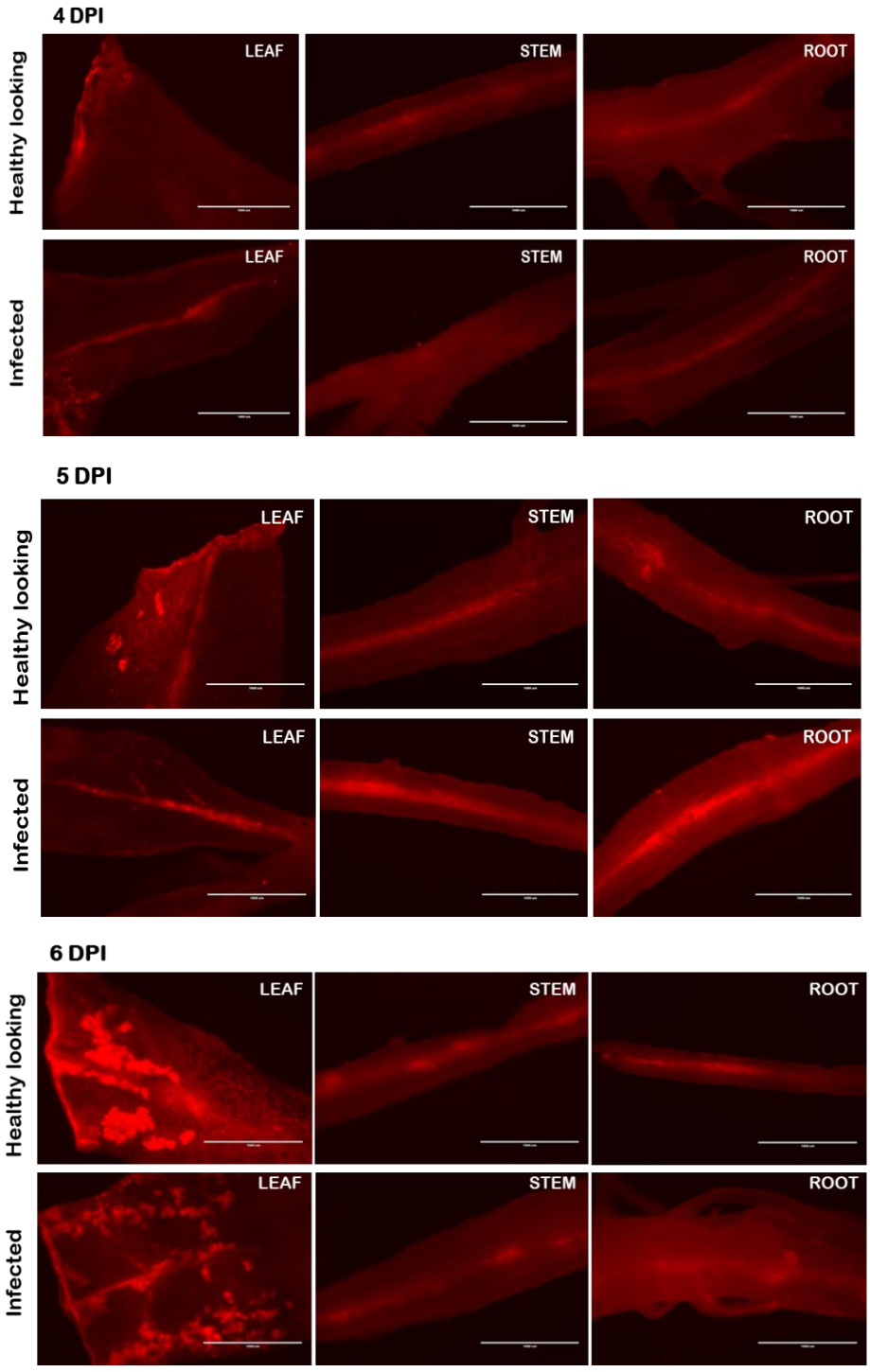
1. Fluorescence images showing colonization of mCherry tagged *R. solanacearum* F1C1, *hrpB* and *hrpB* co-inoculated with wild type F1C1 in grown up tomato plants inoculated by soil drenching method (colonization pattern of these mutants in seedlings of tomato has been shown in chapter 2)



2. Fluorescence images of healthy looking and infected tomato seedlings at different days post inoculation (DPI) inoculated with mCherry marked *R. solanacearum* F1C1 by the leaf clip method (as described in chapter 3)




1 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT
2 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT
3 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT


4 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT
5 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT
6 DPI
Healthy looking
LEAF
STEM
ROOT
Infected
LEAF
STEM
ROOT

Appendix IV

Sequence analysis of polyphosphate gene homologues of F1C1

1. Nucleotide sequence (977 bp) of *ppk1* homolog of *R. solanacearum* F1C1 obtained with oFppk1 & oRppk1 primer pairs (Used for insertion mutation)

>GCCGGATAGTCATTCGTTGGAAACCACCATGTCGACCCTTCCGACCGGCGACCT
GCTCAATCGGGAAGTCGGCATCCTCGAGTTCAACGCGCGCGTGCTCGCACAGGCG
GCCGACCCGGGCGTTCCGCTGATGGAGCGCTTGAAGTTCATCTGCATCGTGTCGA
GCAATCTGGATGAGTTCTTTGAGATCCGCGTCGCCGGGCTGAAGGAACAGATGCG
CGACAACGCCTCGGCCCTCACCCCCGACGGCCTGTCGGTGCAGCAGGCATTTGGC
GTGGTGACGGAGCGGGTACGGCAGCTGGTCGCGCAACAGTATGCCATGCTGCAG
GACGAAATCCTGCCGCTGCTCGAGAAGGAAGGCGTGTTCTTCCACATGACCACCA
ACTGGAACGAAGCCCAGCGCGCCTGGTGCCGCGGCTTCTTCCAGCGCGAGCTAGT
GCCCATCCTGACGCCGATGGCGCTGGACCCGGCCCACCCCTTCCCGCGCGTGCTC
AACAAGAGCCTGAACTTCATCATCGAATTGTCGGGCAAGGATGCCTTCGGGCGCG
AAGCCGAACTGGCCATCGTGCAGGCGCCGCGCGCCCTGCCCCGCCTGGTGCAGAT
GCCGCCGGAGCTGTCGGGGTATCCGTACGGCTTCGTGCTGCTGTCGTCGTTCATG
CAGGGCTTCGTGCACGAGCTGTTTCCGCAGATCGACGTGCATGGCTGCTACCAGT
TCCGCGTCACGCGCAACTCCGACCTGTTCGTCTCCGAAGACGAAATCACCGACCT
GCGCGAAGCGCTGCAAGGCGAACTGTCGACCCGGCACTTCGGCGATGCGGTGCG
GCTGGAGATCGCGCACGACATCTCGCCCGCGCTGGCGCGCCGGCTGCAGCAGGA
ATTCGGCCTGTCCGAAGCCGACTGCTACCGCGTCAAGGGGCCGGTGAACCTGGTG
CGCCTGCTGCAGGTGCCCGACCTGGTGGACCGCCCGATGCTGAAGT

2. Nucleotide sequence (2054 bp) of *ppk1* homolog of *R. solanacearum* F1C1 obtained with oFppk1.A & oRppk1.A primer pairs (Used for antibiotic cassette insertion mutation)

>CCTGCTCAATCGGGAAGTCGGCATCCTCGAGTTCAACGCGCGCGTGCTCGCACA
GGCGGCCGACCCGGGCGTTCCGCTGATGGAGCGCTTGAAGTTCATCTGCATCGTG
TCGAGCAATCTGGATGAGTTCTTTGAGATCCGCGTCGCCGGGCTGAAGGAACAGA
TGCGCGACAACGCCTCGGCCCTCACCCCCGACGGCCTGTCGGTGCAGCAGGCATT
TGGCGTGGTGACGGAGCGGGTACGGCAGCTGGTCGCGCAACAGTATGCCATGCT
GCAGGACGAAATCCTGCCGCTGCTCGAGAAGGAAGGCGTGTTCTTCCACATGACC
ACCAACTGGAACGAAGCCCAGCGCGCCTGGTGCCGCGGCTTCTTCCAGCGCGAG

CTAGTGCCCATCCTGACGCCGATGGCGCTGGACCCGGCCCACCCCTTCCCGCGCG
TGCTCAACAAGAGCCTGAACTTCATCATCGAATTGTCGGGCAAGGATGCCTTCGG
GCGCGAAGCCGAACTGGCCATCGTGCAGGCGCCGCGCGCCCTGCCCCGCCTGGT
GCAGATGCCGCCGGAGCTGTCGGGGTATCCGTACGGCTTCGTGCTGCTGTCGTCG
TTCATGCAGGGCTTCGTGCACGAGCTGTTTCCGCAGATCGACGTGCATGGCTGCT
ACCAGTTCCGCGTCACGCGCAACTCCGACCTGTTCGTCTCCGAAGACGAAATCAC
CGACCTGCGCGAAGCGCTGCAAGGCGAACTGTCGACCCGGCACTTCGGCGATGC
GGTGCGGCTGGAGATCGCGCACGACATCTCGCCCGCGCTGGCGCGCCGGCTGCA
GCAGGAATTCGGCCTGTCCGAAGCCGACTGCTACCGCGTCAAGGGGCCGGTGAA
CCTGGTGCGCCTGCTGCAGGTGCCCGACCTGGTGGACCGCCCGATGCTGAAGTAC
CCGCCGCATGCGCCCGCCGCCGTGAAGGCCGTGGCCGCGTCGTCGAGCATCTTCG
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CGTGCTGGAGCTGCTGCAGCAGGCCGCGCGCGATCCGGACGTGGTCGCCATCAA
GCAGACCGTGTACCGCACCGGCAACGAATCCCCGGTGATGGAGGCGCTGATCAA
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CGTGGTGTACGGCGTGGTCGGCCACAAATGCCACGCCAAGATGCTGCTGGTGGTG
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GCCGGCAAGCGCGCGCGCATCATCGGCAAGATGAACGCGCTGCTGGAGCCGACC
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TGCGCGGCGTGTGCGCGCTCAGGGCCGGCGTGCCGGGGCTGTCGGAACACATCA
GCGTGCGCTCGCTCGTCGGGCGCTTCCTGGAGCATCACCGCGTCTACTACTTCCAT
GCCGGCGGCGAGGAAGTCGTCTATCTGTCCAGCGCCGACTGGATGGACCGCAAC
CTGTTCCGCCGCGTCGAGGTCGCCTTCCCGGTGCTCGATCGCAAGCTGAAGGCGC
GCGTGATCCGCGAAAGCCTGCAGGTGCACCTGCGCGACAACGCCTCGGCCTGGG
TGATGCAGCCGAACGGCTCGTACGTGCGGCGCCAGACCAAGGGCAAGCACGTGC
GCGTGAGCCAGATGGATCTGCTGAACCACTTCTCGC

3. Nucleotide sequence (2356 bp) of *ppk1* homolog of *R. solanacearum* F1C1 obtained with oFppk3 & oRppk3 primer pairs (Used for complementation)

>CAGCAGACGGGGGGTGGGACTCATGGATGGATACGGGTAAATACGCAGGGGGA
TTGTGATGCACGACACAGGGCAACAAAATATGTCTTTGTTGTGTCCTATGCGTGA
CAATTCTAATGCGTCACAAACTTGACACGCGACTATGAAAAAGTGGCCGGATAGT
CATTCGTTGGAAACCACCATGTCGACCCTTCCGACCGGCGACCTGCTCAATCGGG
AAGTCGGCATCCTCGAGTTCAACGCGCGCGTGCTCGCACAGGCGGCCGACCCGG
GCGTTCCGCTGATGGAGCGCTTGAAGTTCATCTGCATCGTGTCGAGCAATCTGGA
TGAGTTCTTTGAGATCCGCGTCGCCGGGCTGAAGGAACAGATGCGCGACAACGC
CTCGGCCCTCACCCCCGACGGCCTGTCGGTGCAGCAGGCATTTGGCGTGGTGACG
GAGCGGGTACGGCAGCTGGTCGCGCAACAGTATGCCATGCTGCAGGACGAAATC
CTGCCGCTGCTCGAGAAGGAAGGCGTGTTCTTCCACATGACCACCAACTGGAACG
AAGCCCAGCGCGCCTGGTGCCGCGGCTTCTTCCAGCGCGAGCTAGTGCCCATCCT
GACGCCGATGGCGCTGGACCCGGCCCACCCCTTCCCGCGCGTGCTCAACAAGAGC
CTGAACTTCATCATCGAATTGTCGGGCAAGGATGCCTTCGGGCGCGAAGCCGAAC
TGGCCATCGTGCAGGCGCCGCGCGCCCTGCCCCGCCTGGTGCAGATGCCGCCGGA
GCTGTCGGGGTATCCGTACGGCTTCGTGCTGCTGTCGTCGTTCATGCAGGGCTTCG
TGCACGAGCTGTTTCCGCAGATCGACGTGCATGGCTGCTACCAGTTCCGCGTCAC
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GAAGCCATCTGCGAAGACGTCCACCACGTGTTCCAGCTGCTGACCGGCACCGGCG
CCCAGGTGAAGCTGCACCACCTGTGGCACTCGCCGTTCACGATGCACGCCAACCT
GATCGAGCACATCCGCGCGGAAGCGCGCGCCGCCCGTGCCGGCAAGCGCGCGCG
CATCATCGGCAAGATGAACGCGCTGCTGGAGCCGACCATCATCGACGAGCTCTAC
AAGGCCTCGCGCGCGGGCGTCAAGATCGACCTGATCGTGCGCGGCGTGTGCGCG

CTCAGGGCCGGCGTGCCGGGGCTGTCGGAACACATCAGCGTGCGCTCGCTCGTCG
GGCGCTTCCTGGAGCATCACCGCGTCTACTACTTCCATGCCGGCGGCGAGGAAGT
CGTCTATCTGTCCAGCGCCGACTGGATGGACCGCAACCTGTTCCGCCGCGTCGAG
GTCGCCTTCCCGGTGCTCGATCGCAAGCTGAAGGCGCGCGTGATCCGCGAAAGCC
TGCAGGTGCACCTGCGCGACAACGCCTCGGCCTGGGTGATGCAGCCGAACGGCT
CGTACGTGCGGCGCCAGACCAAGGGCAAGCACGTGCGCGTGAGCCAGATGGATC
TGCTGAACCACTTCTCGCCGCAAGCCGCCGCGGCCGCGGAAACGGCCGCCGCCGT
CGCAGCGGCCACCGCGACCGAGCCGCCGCGCAAGCCGCCCGCGGAAATCTCCGC
GGGCTGA

4. Nucleotide sequence (827 bp) of *ppk2* homolog of *R. solanacearum* F1C1 obtained with oFppk2 & oRppk2 primer pairs (Used for insertion mutation)

>ATGGGATCGAACGGCAAGACCCCGTTGGACGATTGGCGGTTCGACGGCAGCGG
CAGGTTCAAGATCGCCAAGGCCGACCCCGCGGCCAAGCCCTGCACCACCGGCAC
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CAGGACATCCTCTACGCCGAGCACCACCGCAAGCTGCTGGTGGTCCTGCAGGGCA
TGGACACCTCCGGCAAGGACGGCACCGTGCGCGGCGTGTTCCGGGCCTTCGATCC
GCTAGGCCTGCGCGTGGCCGGCTTCAAGGCCCCGACGCCGCAGGAACTGGCGCG
CGATTTTCTCTGGCGCGTGCATGCCCAGGTGCCGGCGCGCGGCGAGATCGTCATC
TTCAACCGCAGCCACTACGAAGACGTGCTGATCACGCGGGTGCACGGCGACATC
GATGCTGCCGAATGCAAGCGCCGCTATGAGCACATCAGGGCCTTCGAGCGGATG
CTCGAGGAGACCGGCACGACCATCGTCAAATGCTTTCTGCACCTCTCCAGGGACG
AGCAGCGCGCGCGCCTGCAGGAGCGCATCGACGATCCGCACAAGCACTGGAAAT
TCGATCCCGCCGACATCGAAGAGCGCCGATACTGGGACGACTACATGGTGGCCT
ACGAAGACGCCATCAACGCCACCGCGACGCCCGAGGCGCCCTGGCATGTCGTGC
CGGCGGATTCCAAGTCGCACCGCAACCTGATGGTGGCGGAGATCATGCTGCACGT
CCTGGAGCGGATGAAGCCGGAGTATCCGGAGGGGAATCCGGCTTTTGAGGTGGT
GAAGATCGAATG

5. Nucleotide sequence (1189 bp) of *ppx* homolog of *R. solanacearum* F1C1 obtained with oFppx & oRppx primer pairs (Used for insertion mutation)

>CATCACAATCCCCCTGCGTATTTACCCGTATCCATCCATGAGTCCCACCCCCCGT
CTGCTGGCAGCTGTCGACATGGGTTCCAACAGCTTCCGGCTGATGATCGGTCGCG
TCGACGAGACGATCACCGCGAGCGGCAACAGCAGCAGCCAGATCTTCCAGGTCG
ATGCGCTGCGCGAACCGGTCCGGCTGGCGGCCGGGCTTACGCAAGATAAGTACC

TGGACCAGCCCGCGCGCCGTCGCGGCGTCGACGCACTGCGCCGCTTCGGCGACCG
CCTGCGCGATTTCGCGCCCGAGCAGGTGCGCGCGGTGGCGACCAACACGCTGCG
CGTGGCCAAGAACGCGCAGGATTTCCTGATCGAGGCGGAGGCCGCGCTCGGCTT
CCCCATCGAGGTGATCGCGGGCCGCGAAGAGGCCCGCCTGATCTATCTCGGTGCG
TCGCACGATGCGCCGGCGTGCCAGGGCAACCGGCTGGTGGTCGATATCGGCGGC
GGGTCCACGGAGTTCATCATCGGCAACGGCTACAAGCCCAAGCTGATGGAGAGC
CTCTATATCGGTTGCGTTTCGCACAGCCGTCACTTCTTCCCGAATGGCAATGTCGA
CGATTATGCGATGAAGCAGGCCGAGCTGGCCGCGCGTCGCGAGATCCAGGTGCT
GGTGCAGCCGTACCGCACGGCGGGCTGGAAGCAGTCGGTGGGCTCGTCCGGCAC
GGCCCGCGCGCTGGCCGAACTGATCGAGCTCAACGGCTTCAACGACAAGAGCAG
CGAGCACGGCATCACGCGCGAAGGGCTGGAGCGCCTGAAGCGCGCGCTGGTCAA
GGCCGAGAATGCCAACCGCCTCAAGCTGAGCGGCCTCAAGCCGGACCGCATCCC
AGTGCTGCCTGGTGGCCTGTCGATCATGCTGGGCGTCTTCGATGAGCTCGACATC
GACCGCATGGACGTCACCGATGGCGCGCTGCGCCTGGGCGTGCTGTACGACCTGC
TGGGCCGCAGCCATCACGAAGACATGCGCACCGTGACGATCGAGCAGTTCATGC
GCCGCTACAGCGTGGACCGCGCGCAGGCGCACCGGGTGCGGGACGCGGCGACGG
CGCTGCTGTCGCAGTTTCCCGATCCGCCCGATGAGCGGCGCGAGGACAATCTCGC
GCTGCTCGGCTGGGCCGCGAACCTGCACGAGATCGGCATGAGCATC

6. Nucleotide sequence (577 bp) of *ppnk* homolog of *R. solanacearum* F1C1 obtained with oFppnk & oRppnk primer pairs (Used for insertion mutation)

>GCCACTTCGCCCTTCAAGACCGTCGCGCTGGTCGGCCGCTATTCGGCCGCCAAT
ATCGCCGCTCCGCTGCTGGAACTGGCATCGTGCATCGCCGCGCGCGGCCACGATA
TCGTCTTCGAACGGGAAACCGCCCTGAACATCGGGGTCCAGGACTACCCCGCCCT
GCCGCCCGACGAGATGGCGCGCCATGCCGACGTGGCCGTGGTGCTGGGCGGCGA
CGGAACGCTGCTCGGCATCGGGCGCCACCTGGCCGGCGCGTCGGTGCCGGTCATC
GGGGTCAACCACGGCCGGCTCGGCTTCATGACGGACATCCCGTTCGAAGACGTGC
ACGACGTGCTGCCCGACATGCTGGCCGGCCAGTACGAAGCCGAAACCCGCTCGC
TGCTGCAGGCCCAAGTGGTGCGCGATGACGAGACCATCTTCTCCGCCCTGGCCTT
CAACGACGTGGTGGTCAACCGCTCGGGCTTTTCCGGCATGGTCGAGCTGGCCGTC
TCGGTGGACGGCTTCTTCATGTACAACCAGCGCTCGGACGGCCTGATCGTGTCCA
CGCCCACCGGGTCGACGGCCTATGCGCTGT

7. Nucleotide sequence (1156 bp) of *ppnk* homolog of *R. solanacearum* F1C1 obtained with oFppnk3 & oRppnk3 primer pairs (Used for complementation)

>CCGGAATACTTCGACAAGGTGCGCGAGCCCACGGGCTGGCCCTCGGCGATGTAG
CGCTCGATCAGCGTCTTGAGGAGGATGGTGGCGCGTTTGTCCATGATTCATTCGA
TTTTACGCAAATTCACACCGGAAGCGCCGGGTGGCGACAACGTTCCACCTTCAAA
CGCGCGCCGGCCATGGCGACCGTCATATCGATATGGTGTAATGCGCGCATGTCGA
TCTCCCCATCCGCCGTGACTTCCCGCGCGAACGCCACTTCGCCCTTCAAGACCGTC
GCGCTGGTCGGCCGCTATTCGGCCGCCAATATCGCCGCTCCGCTGCTGGAACTGG
CATCGTGCATCGCCGCGCGCGGCCACGATATCGTCTTCGAACGGGAAACCGCCCT
GAACATCGGGGTCCAGGACTACCCCGCCCTGCCGCCCGACGAGATGGCGCGCCA
TGCCGACGTGGCCGTGGTGCTGGGCGGCGACGGAACGCTGCTCGGCATCGGGCG
CCACCTGGCCGGCGCGTCGGTGCCGGTCATCGGGGTCAACCACGGCCGGCTCGGC
TTCATGACGGACATCCCGTTCGAAGACGTGCACGACGTGCTGCCCGACATGCTGG
CCGGCCAGTACGAAGCCGAAACCCGCTCGCTGCTGCAGGCCCAAGTGGTGCGCG
ATGACGAGACCATCTTCTCCGCCCTGGCCTTCAACGACGTGGTGGTCAACCGCTC
GGGCTTTTCCGGCATGGTCGAGCTGGCCGTCTCGGTGGACGGCTTCTTCATGTAC
AACCAGCGCTCGGACGGCCTGATCGTGTCCACGCCCACCGGGTCGACGGCCTATG
CGCTGTCGGCGGGCGGGCCGATCCTGCACCCGGCGCTGTCGGGCCTGGTGCTGGT
GCCGATCGCGCCGCACGCGCTGTCCAACCGGCCCATCGTCATTCCGCACGACGCC
GAGGTCGTCATCCAGGTCACGAGCGGGCGCGACGCCAGCGTCAACTTCGACATG
CAGTCGCTCACGTCGCTGCTGCCGGGCGACCGCATCGTGGTGCGCCGCTCCGAAC
GCACCGTGCGGCTGCTGCATCCGGTCGGCTACAACTACTACGCCACGCTGCGCAA
GAAGCTGCACTGGCACGAGTACCCGACCGAAGACAACCGGCTGTAACGCCAAAA
ACCGAT

Sequence analysis of *hrpG* gene homologue of F1C1

8. Nucleotide sequence (490 bp) of *hrpG* homolog of *R. solanacearum* F1C1 obtained with oG021 & oG022 primer pairs (Used for insertion mutation)

>GCGTACCGAGGCATTCAGTCTGCTGCTGATCGATGCCCAGCAGTTCCGCAGCGC
GGGACAGCTGGTGCTGTCCTGGCGCGAGTGCAACGCCGACATGTGCTGGCCGAC
GCTGGTGTTCGGCCAGTTCGCAGACCGTGAAGACATGGCGCAGGCCTTCGAGGCC
GGCGTCGACGATCTGCTCACCGGCCACTTCACCGCCGAGGAGCTGCGTGCCCGCG
TGCAACGCGTGCTGCGGCGCTCCGAGCAGCCGCGCCAGAACGCCAGCATGCACG
TGGTAGTCGGCCCATACCGGCTCTGCCGCCTGACCCGCACGGCCACGGTGAACGA
AGCGCCGATCCGCCTGACCGCGCGCGAGTTCGCCACGGCCTGGCTGCTGTTCTCG
TCACCGGGCACCTTCCTGTCGCGCCAGCAGATCGCCTCGGCGGTGTGGGGTGCGG
ATGCCAGCATCGTCGAGCGCTCGATCGAGCAGCACATCTACAAGCTGCGCAAGA

Appendix V

List of Publications

Published

[1] Singh, N., **Phukan, T**., Sharma, P. L., Kabyashree, K., Barman, A., Kumar, R., Sonti, R. V., Genin, S., and Ray, S. K. An innovative root inoculation method to study *Ralstonia solanacearum* pathogenicity in tomato seedlings. *Phytopathology*, 108:436–442, 2018, DOI:10.1094/PHYTO-08-17-0291-R.

[2] Kumar, R., Barman, A., **Phukan, T**., Kabyashree, K., Singh, N., Jha, G., Sonti, R. V., Genin, S., and Ray, S. K. *Ralstonia solanacearum* virulence in tomato seedlings inoculated by leaf clipping. *Plant Pathology*, 66:835–841, 2017.

Manuscript under review

[1] **Phukan, T**., Kabyashree, K., Singh, R., Sharma, P. L., Singh, N., Barman, A., Jena, B. R. and Ray, S. K. *Ralstonia solanacearum* pathogenicity in cotyledon stage eggplant seedlings by the leaf clip inoculation method. *Journal of plant pathology* (under review).

Manuscript under preparation

[1] **Phukan, T**. *et al. Ralstonia solanacearum* load in inoculated tomato seedlings is not enough to define the wilt disease in the seedlings.

[2] **Phukan, T**. *et al.* Role of *ppk1* in the virulence of *Ralstonia solanacearum* F1C1 in tomato seedlings.

Published in conferences/proceedings

[1] **Phukan, T**., Kabyashree, K. Singh, R., and Ray, S. K. A study on *Ralstonia solanacearum* F1C1 pathogenicity in Eggplant (Brinjal) seedlings. In *International Symposium on* "*Technical Intervention in Microbial Resource*", page no. 12, Tezpur University, Tezpur, Assam, 2018 (Oral presentation).

[2] **Phukan, T**. and Ray, S. K. A polyphosphate kinase (*ppk1*) mutant of *Ralstonia solanacearum* F1C1 is virulence and motility deficient. In *International Symposium on "Plant Biotechnology for Crop Improvement"*, page no. 46, IIT, Guwahati, Assam, 2017.

[3] **Phukan, T**. and Ray, S. K. A genetic approach to understand the role of polyphosphate metabolism in *Ralstonia solanacearum* pathogenicity in tomato seedlings. In *58th Annual Conference of Association of Microbiologists of India (AMI-2017) & International Symposium on "Microbes for Sustainable Development: Scope & Applications"*, page no. 314, BBAU, Lucknow, UP, 2017.

[4] Kumar, R., Barman, A., **Phukan, T**., Kabyashree, K., Singh, N. and Ray, S. K. A simple infection method to study Virulence of *Ralstonia solanacearum* using tomato seedlings, In *6th International Bacterial Wilt Symposium (IBWS-2016)*, page no. 105, LIPM, Toulouse, France, 2016.

[5] Kabyashree, K., Kumar, R., **Phukan, T**. and Ray, S. K. Evidence indicating avoidance of root colonization by *Ralstonia solanacearum* F1C1 strain in tomato seedling infection. In *International Seminar on "MCB75: from molecules to organisms*", IISc, Bangalore, page no., 2015.

Plant Pathology (2017) **66,** 835–841 Doi: 10.1111/ppa.12628

Ralstonia solanacearum virulence in tomato seedlings inoculated by leaf clipping

R. Kumar[ab†], A. Barman[a†], T. Phukan[a], K. Kabyashree[a], N. Singh[a], G. Jha[b], R. V. Sonti[c], S. Genin[d] and S. Kumar Ray[a*]

[a]*Dept of Molecular Biology and Biotechnology, Tezpur University, Tezpur, 784028, Assam;* [b]*National Institute of Plant Genome Research, Aruna Asaf Ali Road, New Delhi, 110067;* [c]*Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad, 500007, Andhra Pradesh, India; and* [d]*LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, F-31326, France*

Ralstonia solanacearum is a phytopathogenic bacterium that colonizes the xylem vessels of host plants leading to a lethal wilt disease. Although several studies have investigated the virulence of *R. solanacearum* on adult host plants, infection studies of this pathogen on the seedling stages of hosts are less common. In a preliminary observation, inoculation of *R. solanacearum* F1C1 on 6- to 7-day-old tomato seedlings by a simple leaf-clip strategy resulted in a lethal pathogenic condition in seedlings that eventually killed these seedlings within a week post-inoculation. This prompted testing of the effect of this inoculation technique in seedlings from different cultivars of tomato and similar results were obtained. Colonization and spread of the bacteria throughout the infected seedlings was demonstrated using *gus*-tagged *R. solanacearum* F1C1. The same method of inoculating tomato seedlings was used with *R. solanacearum* GMI1000 and independent mutants of *R. solanacearum* GMI1000, deficient in the virulence genes *hrpB*, *hrpG*, *phcA* and *gspD*. Wildtype *R. solanacearum* GMI1000 was found to be virulent on tomato seedlings, whereas the mutants were found to be non-virulent. This leaf-clip technique, for inoculation of tomato seedlings, has the potential to be a valuable approach, saving time, space, labour and costs.

Keywords: bacterial wilt, leaf-clip inoculation, *Ralstonia solanacearum*, tomato seedling infection

Introduction

Ralstonia solanacearum is a Gram-negative phytopathogenic bacterium that causes a lethal wilt disease in more than 450 plant species belonging to 54 botanical families of angiosperms (Hayward, 1991; Elphinstone, 2005; Wicker *et al.*, 2007; Genin, 2010). The bacterium, which was previously known as *Pseudomonas solanacearum*, has a wide geographical distribution. Because of its complex pathogenicity leading to a lethal wilting disease in many different plants, several laboratories around the world are engaged in research related to this bacterium (Mansfield *et al.*, 2012).

The bacterium is soilborne and has a wide genetic diversity among the strains that are described under the *R. solanacearum* species complex (RSSC). In the presence of a suitable host, the bacterium invades through roots and colonizes the xylem. From the xylem it spreads through the entire host plant before causing wilting and death of the plant. Molecular genetic studies of *R. solanacearum* have provided valuable insights into its pathogenicity mechanisms, such as different protein secretion systems (Liu *et al.*, 2005; Poueymiro & Genin, 2009), cell-to-cell signalling molecules (Flavier *et al.*, 1997a,b; Kai *et al.*, 2015), exopolysaccharides (Cook & Sequeira, 1991; Huang & Schell, 1995) and two-component regulatory systems (Clough *et al.*, 1997; Aldon *et al.*, 2000). However, several questions about pathogenesis mechanisms of this bacterium still remain to be answered. For example, although it is known that the bacterium colonizes the whole plant before causing wilting, the switch from the colonization phase to virulent phase inside the plant is not well understood. In addition, the mechanism through which the pathogen avoids infecting host tissues immediately after its entry and hold backs from colonizing the vascular system before causing wilting are not known, nor is the influence of other plant-associated bacteria on the *R. solanacearum* infection mechanism.

In most laboratories, infection studies of *R. solanacearum* on tomato host plants use one of two methods: soil drenching or stem inoculation. Both these methods require fully grown tomato plants for the infection study. Prior to infection, tomato seedlings are first grown for *c.* 45 days to the plantlet stage in a greenhouse at optimum temperature and humidity. Completion of an infection study using either of these two strategies takes at least 60 days. Furthermore, because plantlets are grown in pots containing soil, association of the plantlets with soil microbiota including other bacteria cannot be avoided. These soil microbiota may

*E-mail: suven@tezu.ernet.in
†These authors contributed equally to the work.

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interfere with the infection process as well as subsequent observations, meaning that disease profiles may not be due to *R. solanacearum* inoculation alone.

There have been previous studies of bacterial wilt progression using tomato seedlings (Pradhanang *et al.*, 2000). In the authors' laboratory, a study of infection by *R. solanacearum* F1C1 (Kumar *et al.*, 2013) by a root-inoculation method, under axenic conditions, demonstrated its pathogenicity in 6–7-day-old tomato seedlings of tomato cultivar S-22 (Evergreen Seed) (Kumar, 2014). However, the presence of disease symptoms could not be observed in seedlings of other tomato cultivars using the same method. In adult rice plant leaves, leaf-clip inoculation of *Xanthomonas oryzae* pv. *oryzae*, a vascular pathogen, has been regularly used to study bacterial leaf blight of rice (Kauffman *et al.*, 1973; Ray *et al.*, 2000). Therefore, the aim of the present study was to determine the effectiveness of a similar leaf-clip method to inoculate 6–7-day-old tomato seedlings with *R. solanacearum* for rapid investigations of virulence. A *gus*-tagged strain (encodes *β*-glucuronidase) of *R. solanacearum* F1C1 was also inoculated into seedlings by the same technique to confirm colonization of the infected seedlings. To validate potency of the technique, *R. solanacearum* GMI1000 and mutants deficient for important virulence genes (*hrpB*, *hrpG*, *phcA* and *gspD*) were used.

Materials and methods

Bacterial strains and growth media

Bacterial strains, plasmids and their specific characteristics used in this study are listed in Table 1. *Ralstonia solanacearum* was grown in BG medium (Boucher *et al.*, 1985) supplemented with 0.005% 2,3,5-triphenyl tetrazolium chloride (TZC; Himedia) and 0.5% glucose. *Escherichia coli* was grown in LB medium (Bertani, 1952) at 37 °C, and 1.5% agar was added for LB agar medium. Concentrations of antibiotics used were as follows: ampicillin (Amp; 50 μg mL^{-1}), spectinomycin (Spc; 50 μg mL^{-1}) and rifampicin (Rif; 50 μg mL^{-1}). All antibiotics were bought from Himedia.

Germination of tomato seedlings and transfer to microcentrifuge tubes

For the germination process, seeds of tomato cultivar NS812 (Namdhari Seeds) were pre-soaked in sterile distilled water and then germinated on sterile wet tissue paper in a plastic tray (Fig. 1a,b) in a growth chamber (Orbitek) maintained at 28 °C, 75% relative humidity with a 12 h photoperiod. Distilled water was sprinkled regularly to sustain the germination process. Each 7-day-old seedling was transferred to a sterile 1.5–2 mL centrifuge tube containing 1–1.5 mL distilled water (Fig. 1c).

Inoculation of tomato seedlings with *R. solanacearum* F1C1 and some nonpathogenic bacteria

Tomato seedlings of cultivar NS812 (7 days old) in microfuge tubes were inoculated with *R. solanacearum* F1C1 or the nonpathogenic bacteria *Bacillus subtilis*, *Escherichia coli* or *Pseudomonas putida* by a leaf-clip method described below.

Table 1 Bacterial strains used in this study

Strain	Characteristics	Reference
Ralstonia solanacearum		
F1C1	Wildtype virulent strain, phylotype I in India, isolated from a wilted chilli plant collected from a field at Tezpur University, Tezpur, India	Kumar *et al.* (2013)
TRS1001	Rif^r, Vir^+ strain derived from F1C1	Kumar (2014)
TRS1002	GUS +ve, Vir^+, Rif^r, Spc^r, derived from TRS1001 after Tn*5gusA11* insertion in an unknown locus in the genome	Kumar (2014)
GMI1000	Wildtype, phylotype I	Salanoubat *et al.* (2002)
GMI1525	*hrpB* mutant of GMI1000, deficient in type III protein secretion system and in virulence	Genin *et al.* (1992)
GMI1755	*hrpG* mutant of GMI1000, deficient in type III protein secretion system and in virulence	Valls *et al.* (2006)
GMI1605	*phcA* mutant of GMI1000, deficient in exopolysaccharide and in virulence	Genin *et al.* (2005)
GRS465	*gspD* mutant of GMI1000, deficient in type II protein secretion system and in virulence	Liu *et al.* (2005)
GRS445	*prhG* mutant of GMI1000, mild virulence deficiency	Plener *et al.* (2010)
Escherichia coli		
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	Laboratory collection
TP003	S17-1 carrying Tn*5gusA11* in a suicide plasmid	Laboratory collection
Bacillus subtilis		Laboratory collection
Pseudomonas putida		Laboratory collection

Preparation of bacterial inoculum

A loopful of bacteria from a colony of wildtype *R. solanacearum* F1C1, freshly grown on a plate containing BG agar, was added to 50 mL BG broth and incubated at 28 °C in a shaking incubator (Orbitek). After 24 h, cultures were centrifuged at 3155 ***g*** for 15 min. Pellets were resuspended in sterile distilled water to obtain a bacterial concentration of *c.* 10^9 CFU mL^{-1}.

Pseudomonas putida, *E. coli* and *B. subtilis* were grown as described above, but *E. coli* and *B. subtilis* were cultured in LB at 37 °C. After 24 h the inoculum was prepared as for *R. solanacearum* F1C1.

Inoculation by leaf clipping

A pair of sterile stainless steel scissors was dipped in the bacterial suspension (*c.* 10^9 CFU mL^{-1}) every time prior to clipping off one-third of a leaf from the tip region in each seedling. Forty tomato seedlings were inoculated for each bacterial species. As a control, 40 seedlings were 'mock-inoculated' with sterile distilled water in the same way. Inoculated seedlings were transferred to a growth chamber (Orbitek) maintained at 28 °C, 75% relative humidity and a photoperiod of 12 h. Seedlings were analysed and observations were recorded each day until 10 days post-inoculation.

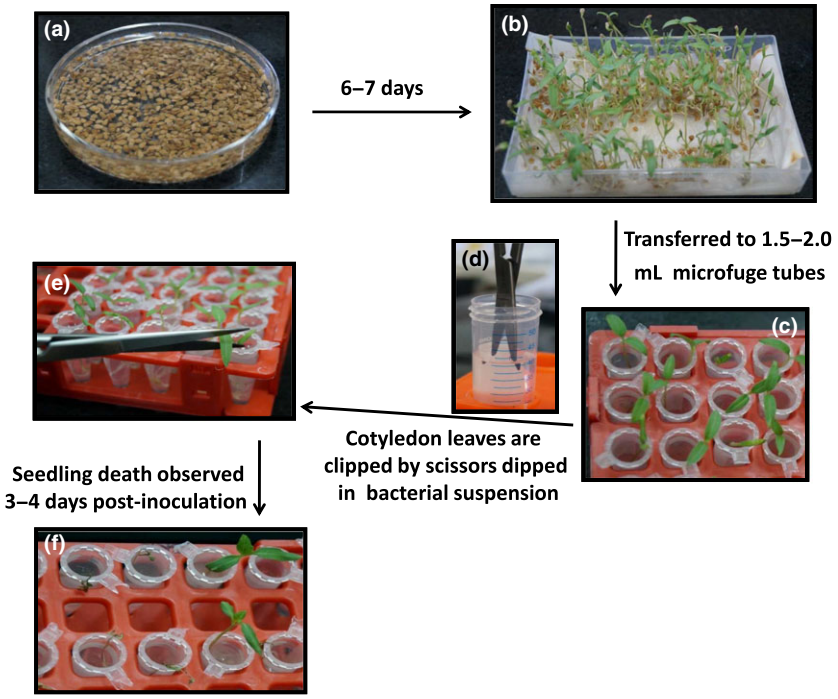

Figure 1 A schematic diagram showing the leaf-clip inoculation of tomato seedlings with *Ralstonia solanacearum*. (a) Tomato seeds are pretreated with sterile distilled water. (b) Imbibed seeds are transferred to a sterile and wet tissue paper bed, spread out and allowed to germinate in a growth chamber (28 °C; 75% RH) until the seedling stage. (c) 6–7-day-old seedlings are each transferred to sterile microfuge (1.5–2 mL) tubes containing 1–1.5 mL sterile distilled water. (d) A pair of sterile scissors (stainless steel) is dipped in inoculum. (e) After every dipping, one-third of each cotyledon leaf, from the tip, is clipped off. Inoculated tomato seedlings are subsequently transferred to a growth chamber maintained at optimum conditions (28 °C; 75% RH; 12 h light/12 h dark). (f) After 7–9 days, 70–80% of infected tomato seedlings are generally observed to have died. [Colour figure can be viewed at wileyonlinelibrary.com]

Inoculation of different cultivars of tomato seedlings with *R. solanacearum* F1C1

Seedlings of eight commercially available cultivars of tomato were tested by leaf clip inoculation with *R. solanacearum*. These cultivars were Badshah (Nunhems), Akhilesh, PHS-7799 and Vijay (Param Hybrid Seeds), NS812 (Namdhari Seeds), SHV22 (Sahavi Hybrid Seeds), Navoday (Durga Seed Farm) and Classic (Classic hybrid seeds). The seeds of all these cultivars were germinated and prepared for inoculation as described above.

Ralstonia solanacearum F1C1 inoculum was prepared as described above. For each cultivar, 40 seedlings were inoculated with *R. solanacearum* F1C1 by the leaf-clip method and 40 seedlings were treated with sterile distilled water as controls. The inoculated seedlings were observed up to 10 days post-inoculation. The experiment was repeated at least three times.

Inoculation of tomato seedlings with different titres of *R. solanacearum* F1C1

Tomato seedlings (NS812) were inoculated with different titres of *R. solanacearum* F1C1 by leaf clipping to determine the effect of dilution on the progression of disease symptoms on the inoculated seedlings. A fresh culture of *R. solanacearum* F1C1 was centrifuged, as described previously, and resuspended in sterile distilled water to give a concentration of *c.* 10^9 CFU mL^{-1}. The bacterial suspension was serially diluted in sterile distilled water to obtain 10^8, 10^7, 10^6 and 10^5 CFU mL^{-1} of *R. solanacearum* F1C1. The methodology and evaluation of the infection process was as described above.

Creation of *gus*-tagged *R. solanacearum*

Ralstonia solanacearum F1C1 was tagged with the reporter gene *gus* by biparental mating between *E. coli* TP003 and *R. solanacearum* TRS1001 (a spontaneous rifampicin-resistant strain of F1C1) using small modifications to the method of Ray *et al.* (2000). The former carries a constitutively expressed *gus* gene in a Tn*5* transposon (Wilson *et al.*, 1995), which is a mini-transposon and is part of a conjugable suicide plasmid. The donor (TP003), grown in LB + Spc at 37 °C for 24 h, and the recipient (TRS1001), grown in BG + Rif at 28 °C for 24 h, were each centrifuged at 3155 *g* (5804R; Eppendorf) for 10–15 min. The supernatants were removed and the pellets were resuspended in an equal volume of sterile water. Bacteria were pelleted again by repeating the centrifugation procedure. The supernatants were discarded and the pellets suspended in one tenth original culture volume of sterile water. Fifty microlitres of the suspended TRS1001 culture was put onto BG agar in a Petri dish and left inside a laminar air flow cabinet for 1 h to dry. TP003 cells were removed with a sterile toothpick and mixed with TRS1001. Both the donor and the recipient were spotted separately onto BG agar as controls. The plates containing bacteria were incubated at 28 °C for 48 h. Subsequently, the mixed bacteria were removed by scraping the agar surface with a sterile loop and the cells were suspended in 150 µL sterile water, vortexed and spread onto BG+Rif+Spc plates. After 48 h, the transconjugants were checked for ampicillin sensitivity by streaking on BG+Rif+Spc and BG+Rif+Spc+Amp plates separately.

Colonies sensitive to ampicillin were checked for the presence of *gus* activity. In a 1.5 mL microcentrifuge tube, a loopful of bacteria from an ampicillin-sensitive colony was added to 120 µL sterile water and vortexed for 30 s. One microlitre of X-gluc (100 mg mL^{-1} dissolved in dimethyl formamide) was added and incubated at 37 °C for 30 min. Appearance of blue colour in the suspension confirmed the presence of *gus* in the new strain (TRS1002). The wildtype F1C1 was negative for the *gus* assay, as anticipated.

GUS staining of tomato seedlings

About 20 seedlings (6–7 days old) of tomato cv. NS812 were inoculated with *gus*-tagged *R. solanacearum* (TRS1002) by leaf

clipping; as a control, *R. solanacearum* F1C1 was inoculated using the same procedure.

The tomato seedlings were stained with 0.5 mg mL^{-1} X-gluc (in 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.1% Triton X-100) following a modified method of Jefferson *et al.* (1987): the seedlings were washed several times with sterile water to remove surface-adhered bacteria and were sterilized using $HgCl_2$ (0.04%) and 70% ethanol. Surface-sterilized seedlings were transferred to 2 mL microcentrifuge tubes containing X-gluc solution and left for 24 h at 37 °C. Seedlings were then washed with sterile water and further immersed in 70% ethanol for 3–4 h to improve visualization of the GUS staining.

Studies on *R. solanacearum* virulence functions

Forty seedlings (6–7 days old) of tomato cultivar Marmande (LIPM) were inoculated by the leaf clipping method with wildtype *R. solanacearum* GMI1000 (Salanoubat *et al.*, 2002) and some of its derivative strains that carry independent mutations in the *hrpB*, *hrpG*, *gspD* and *phcA* genes. The controls were 40 seedlings that were clipped but not inoculated with bacteria, and 40 seedlings that were not clipped or inoculated. A Kaplan–Meier survival probability [S(t)] curve was calculated for seedlings inoculated with each strain (Kaplan & Meier, 1958). Additionally, the tomato seedlings were inoculated with another strain of GMI1000 that harboured a mutation in the *prhG* gene.

Results

Ralstonia solanacearum F1C1 infects tomato seedlings upon inoculation by leaf clipping

Susceptibility of the tomato seedlings to *R. solanacearum* using a leaf-clip inoculation method was investigated. The disease symptoms in the seedlings were observed from the third day post-inoculation onwards. By the tenth day post-inoculation, about 70–80% of seedlings inoculated with *R. solanacearum* were dead. The virulence was significant and repeatable.

To check whether the death of the seedlings was due to the pathogenicity of *R. solanacearum* and not merely due to the presence of bacteria, seedlings were inoculated with *B. subtilis*, *E. coli* and *P. putida*, known to be nonpathogenic to plants. Seedlings were not affected by inoculation with the nonpathogenic bacteria, suggesting that the seedling death was due to the pathogenicity of *R. solanacearum* F1C1.

To check susceptibility of other cultivars of tomato towards F1C1, eight cultivars of tomato were tested and all exhibited susceptibility towards F1C1 by this method of inoculation. Out of these, three cultivars, Namdhari, Sahavi and Durga, were inoculated with F1C1 three times by the same procedure and infection, leading to death, was observed (Fig. 2) in every experiment.

To test the sensitivity of tomato seedlings further, 7-day-old seedlings were inoculated with serially diluted F1C1 inoculum. Disease symptoms were apparent for all the titres of F1C1 from the third day post-inoculation onwards. The number of seedlings killed was similar for 10^9 CFU mL^{-1} as for 10^6 CFU mL^{-1} (Figure S1; Table S1), but the susceptibility of seedlings was reduced at a bacterial concentration of 10^5 CFU mL^{-1}. However, it was apparent that even 10^5 CFU mL^{-1} could elicit pathogenicity symptoms similar to the culture of F1C1 at a titre of 10^9 CFU mL^{-1} when inoculated through leaf clipping.

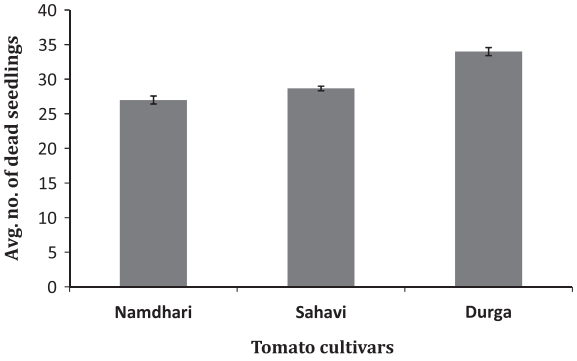

Figure 2 *Ralstonia solanacearum* F1C1 is virulent to seedlings of different tomato cultivars when inoculated via leaf clipping. Bar chart shows results of virulence assay after 40 seedlings of three different tomato cultivars, Namdhari, Sahavi and Durga, were inoculated by leaf clipping with *R. solanacearum* F1C1 (10^9 CFU mL^{-1}). Means and standard deviations of three experiments are shown.

To confirm that the death of seedlings was caused by colonization by *R. solanacearum*, 7-day-old tomato seedlings were inoculated by leaf clipping with *gus*-tagged *R. solanacearum* TRS1002. TRS1002 was virulent like the wildtype F1C1, suggesting that *gus* insertion had not affected the virulence of the tagged strain. GUS staining of the seedlings was performed on the fifth day post-inoculation as well as the control seedlings to analyse *R. solanacearum* colonization in the seedlings. The seedlings inoculated with TRS1002 stained blue while the control seedlings (inoculated with untagged *R. solanacearum* F1C1) did not show any colour (Fig. 3). The blue colour observed in the stem indicated the presence of bacteria in the seedlings. Bacteria were also detected towards the root region of the seedlings, indicating that bacteria inoculated via leaf clipping could colonize and spread to lower parts of the seedling. However, GUS staining was non-uniform throughout the seedlings.

Studies on *R. solanacearum* virulence functions

To further evaluate the leaf-clip inoculation technique for studying virulence functions of *R. solanacearum*, seedlings (7 days old) of tomato cultivar Marmande were inoculated with wildtype *R. solanacearum* GMI1000 and some of its derivative strains that carry independent mutations in the *hrpB*, *hrpG*, *gspD* and *phcA* genes. The Kaplan–Meier survival curve for the virulence assay is given in Figure 4. The four mutants exhibited significantly less virulence on tomato seedlings in comparison to the wild type (calculated via a log-rank test, $P < 0.05$). The additional *prhG* mutant exhibited higher

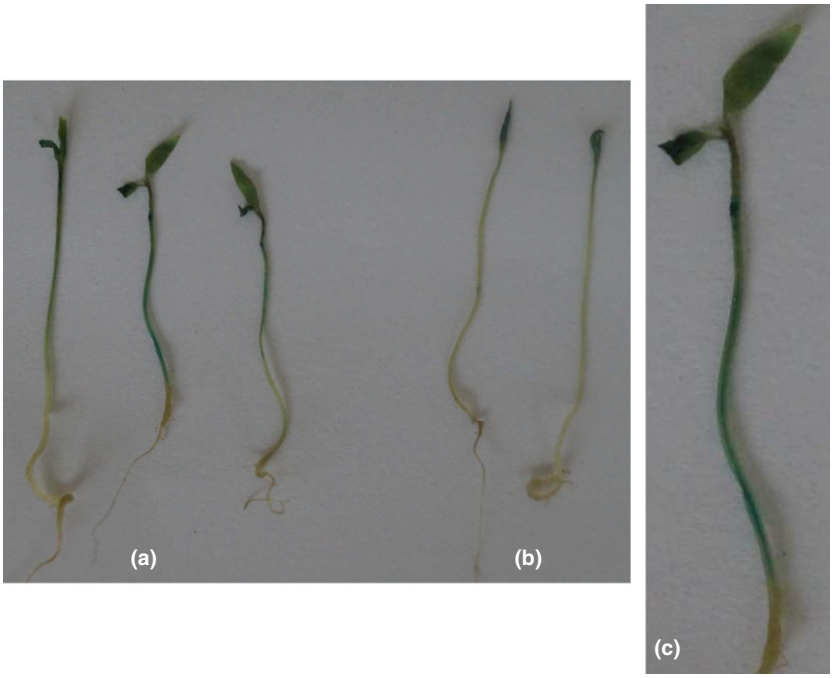

Figure 3 GUS staining of infected tomato seedlings 5–6 days after inoculation by leaf clipping with *Ralstonia solanacearum* TRS1002 (*gus*-tagged). (a) Blue staining of infected seedlings was non-uniform, with clumps of blue stain throughout the seedlings. (b) GUS staining of control seedlings (without TRS1002 inoculation), which remain colourless. (c) An enlarged image of an infected seedling showing blue stain due to GUS activity. [Colour figure can be viewed at wileyonlinelibrary.com]

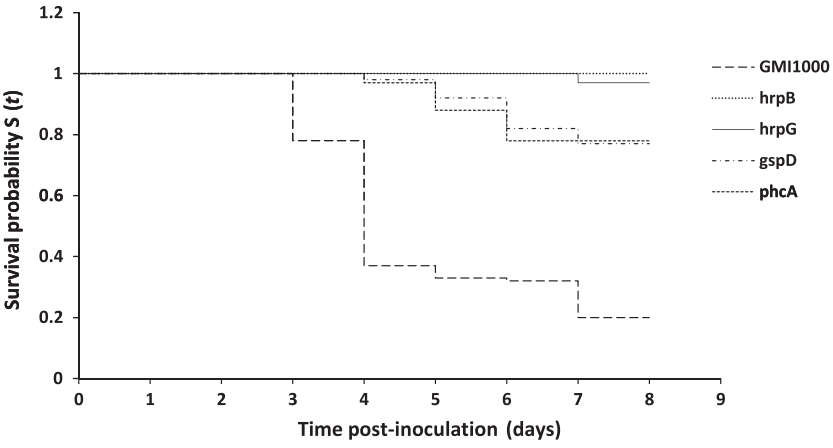

Figure 4 Kaplan–Meier survival probability [S(t)] curve for a virulence assay performed on tomato seedlings inoculated by leaf clipping with wildtype *Ralstonia solanacearum* GMI1000 and four mutants. The GMI1000 mutants carried independent mutations *hrpB*, *hrpG*, *gspD*, *phcA* and exhibited virulence deficiency on tomato seedlings. All mutants were found to be significantly deficient in virulence compared to the wildtype GMI1000 ($P < 0.05$; log-rank test).

virulence on the tomato seedlings (data not shown) than the other four mutants.

Discussion

Ralstonia solanacearum causes a lethal wilt disease in numerous plant species. In this study a leaf-clip method of inoculation of 6–7-day-old tomato seedlings has been described to study the pathogenicity of this bacterium. This strategy of inoculation was successfully used to study the pathogenicity of two different wildtype *R. solanacearum* strains (F1C1 and GMI1000) against different tomato cultivars in two different laboratories. The advantages of this inoculation technique are as follows: (i) the study takes *c.* 20 days starting from seed germination to completion of the infection process; (ii) this technique may be used to screen large numbers of *R. solanacearum* strains or mutants in a limited space; (iii) this technique is able to reproduce the infection assay outcomes previously reported for several virulence mutants, such as *hrpB*, *hrpG*, *gspD*, *phcA* or *prhG*, using the more natural soil-drenching inoculation method; (iv) the infection process avoids interference by other bacteria/microbiota from the soil during the infection process; and (v) this strategy might prove advantageous for colonization studies of *R. solanacearum* in tomato seedlings.

Ralstonia solanacearum is a vascular pathogen and so, for pathogenicity studies, most investigators choose to infect adult plant hosts with well-developed xylem vessels. Thus, there is no clear mention in previous literature about the pathogenicity of this bacterium towards

seedlings (possessing vascular tissues at early stages of development). Therefore, the present study of the pathogenicity of *R. solanacearum* towards 6–7-day-old tomato seedlings inoculated by leaf clipping is important. It has been observed that *R. solanacearum* elicited disease symptoms in the seedlings at the site of inoculation and these symptoms gradually spread downward to the root region. This indicates that the bacterium is capable of causing disease at the site of entry, soon after invading the tissue, and may not have to colonize the entire seedling for the development of the disease symptoms in the seedlings. Further, this study also suggests that the bacterium can move from leaves to other parts of the seedling downward, comparable to the migration of *X. oryzae* pv. *oryzae* in its host (Chatterjee *et al.*, 2003).

The virulence study on seedlings documented here might be helpful in understanding the *in planta* gene expression pattern of *R. solanacearum*. Strains that carried independent mutations in the *hrpB*, *hrpG*, *gspD* and *phcA* genes were shown to have reduced virulence on the seedlings. This result is in agreement with previous studies of these mutants on tomato plants (Brumbley & Denny, 1990; Genin *et al.*, 1992; Brito *et al.*, 1999; Vasse *et al.*, 2000; Lin *et al.*, 2008). In addition, the *prhG* mutant exhibited higher virulence on the tomato seedlings (data not shown) than the other four mutants, confirming the findings from a previous virulence study on mature tomato plants that used a soil drenching inoculation method (Plener *et al.*, 2010). Taken together, these observations suggest that the leaf-clip inoculation technique on seedlings is able to reproduce disease symptoms similar to those previously observed with virulence mutants on mature plants. It can therefore be considered as a rapid and sensitive inoculation strategy to study different virulence functions of this pathogenic bacterium.

There have been studies identifying genes that are expressed when the bacterium is inside the plant (Vasse *et al.*, 2000; Jacobs *et al.*, 2012; Monteiro *et al.*, 2012). It is known that, apart from the pathogen, many endophytes are also present inside the vascular tissues of the plant (Achari & Ramesh, 2014; Upreti & Thomas, 2015). The role of these endophytes in modifying bacterial wilt symptoms is unknown, but cannot be overlooked. It might be that several of the genes induced in the pathogen after entering the host tissue are actually required for its coexistence with the endophytes already present inside the xylem; the seedling assay described here may prove useful in elucidating this. In addition, the leaf-clip method could be used for coinoculations to study the synergistic or antagonistic effects of endophytic bacteria on the bacterial wilt pathogen.

It is important to note that there is a recent report that showed infection of tomato seedlings by *R. solanacearum* (Thomas & Upreti, 2014). However, unlike this previous study, the present method has avoided contact of the tomato seeds with the soil, starting from seed germination to infection assay completion. Thus, this approach minimizes the possibility of colonization of the seeds by soil bacteria other than endophytes already associated with them.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Effect on tomato seedlings when leaf-clip inoculated with different titres of *Ralstonia solanacearum* F1C1.

Table S1. Average number of tomato seedlings killed upon inoculation with *Ralstonia solanacearum* by leaf clipping.

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Bacteriology

e-Xtra*

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

N. Singh, T. Phukan, P. L. Sharma, K. Kabyashree, A. Barman, R. Kumar, R. V. Sonti, S. Genin, and S. K. Ray†

First, second, third, fourth, fifth, sixth, and ninth authors: Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India; seventh author: Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, Andhra Pradesh, India; and eighth author: LIPM, Université de Toulouse, INRA, CNRS, F-31326 Castanet-Tolosan, France.

Present address of A. Barman: Department of Biotechnology, Pandu College, Guwahati 781012, Assam, India.

Present address of R. Kumar: National Institute of Plant Genome Research, Aruna Asaf Ali Road, New Delhi 110067, India.

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ABSTRACT

In this study, we report *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- to 7-day-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 2 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, and cost as well as the required quantity of bacterial inoculum. Thus, the newly developed assay is 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.

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 (Elphinstone 2005; Genin 2010; Hayward 1991; Wicker et al. 2007). The pathogen exceptionally has a wide host range (Coutinho et al. 2000; Jiang et al. 2016; Ozaki and Watabe 2009; 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 the term "species-complex" in regard to this pathogenic bacterium (Fegan and 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 and type III) essential for virulence functions in this bacterium have been uncovered (Coll and Valls 2013; Genin and Boucher 2002; Monteiro et al. 2012a; Vasse et al. 2000). In planta gene expression studies of *R. solanacearum* (Ferreira et al. 2017; Jacobs et al. 2012; Puigvert et al. 2017) have provided several clues regarding the adaptive responses of the bacterium within its host. Recently, the roles of diffusible quorum-sensing molecules and extracellular DNAses as well as biofilm formations were reckoned to be important for its pathogenic interaction with host (Hikichi et al. 2017; Kai et al. 2015; Mori et al. 2016; Tran et al. 2016). Despite a wealth of exciting findings in regard to *R. solanacearum* biology (Guidot et al. 2014; Hikichi et al. 2017; Marchetti et al. 2010; Peyraud et al. 2016; Remigi et al. 2014), knowledge pertaining to mechanism of its entry into a 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 considered an attractive model for investigating fundamental aspects of the plant–pathogenic bacteria interaction as well as pathogen–host adaptations (Coll and Valls 2013; Ferreira et al. 2017; Genin 2010; Genin and Boucher 2002). Grown tomato plants are generally utilized as suitable model hosts, where the pathogen is inoculated either by soil drenching or by stem inoculation. In addition, *Arabidopsis thaliana, Phaseolus vulgaris* (a distant host plant), and *Mimosa pudica* (a nonhost plant) are other model plants which have been utilized for understanding virulence, host resistance, and evolution of *R. solanacearum* (Deslandes et al. 2002; Guidot et al. 2014; Marchetti et al. 2010; Yang and Ho 1998;). Host plants raised in soil come in contact with soilborne microbes and these associations, in turn, could modulate their fitness (Feau and Hamelin 2017). Therefore, a resident microbial population can affect inferences of *R. solanacearum* virulence assays conducted on such hosts. Furthermore, growing and maintaining large numbers of adult plants requires ample amounts of space and 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 a few mutants of *R. solanacearum* (Macho et al. 2010).

Seedling stages of tomato plants have been used for studying *R. solanacearum* pathogenicity on a number of occasions (Artal

†Corresponding author: S. K. Ray; E-mail: suven@tezu.ernet.in

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*The *e-Xtra* logo stands for "electronic extra" and indicates that eight supplementary figures and eight supplementary tables are published online.

et al. 2012; Kumar 2014; Kumar et al. 2017; Monteiro et al. 2012a; Park et al. 2007; Pradhanang et al. 2000). In fact, there is an interesting recent report on *R. solanacearum* root infection in early stages of *A. thaliana* under gnotobiotic condition (Lu et al. 2018). 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). Because *R. solanacearum* naturally infects its plant host via the root, its behavior inside the plant may distinctly differ when the pathogen is introduced through other means such as leaf clipping. Therefore, devising an efficient root inoculation method devoid of such constraints was imperative. In this context, here, we report an innovative method of *R. solanacearum* root inoculation into 6- to 7-day-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 requiring 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 the 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) and derivative mutant strains as well as *Pseudomonas putida* was BG (Bacto agar-glucose) 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 Luria-Bertani (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 (50 μg ml^{-1}), ampicillin (50 μg ml^{-1}), rifampicin (50 μg ml^{-1}), and gentamycin (50 μg ml^{-1}). All media components and antibiotics used in this work were bought from Himedia.

Germination of tomato seedlings for inoculation. Tomato seed of different cultivars recruited in this study were presoaked in sterile distilled water for 2 days. This was followed by spreading the seed on sterile wet tissue paper in a plastic tray and allowing them to germinate in a growth chamber (Orbitek) maintained at 28°C, 75% relative humidity (RH), and a 12-h photoperiod. Sterile distilled water was sprinkled regularly to sustain the germination process for 6 to 7 days. Age of the seedling was defined from the day the seed were kept for germination on the wet tissue paper bed.

Preparation of bacterial inoculum. *R. solanacearum* F1C1 was streaked on a BG agar plate. Freshly grown *R. solanacearum* (F1C1) colonies were added to 50 ml of BG broth with a sterile loop and allowed to grow in a shaking incubator (Orbitek) maintained at 28°C and 150 rpm. After 24 h, bacterial cultures were centrifuged at 4,000 rpm (3,155 × *g*) for 15 min at 4°C. Bacterial pellets were resuspended in an equal volume of sterile distilled water to obtain a concentration of approximately 10^9 CFU ml^{-1}. *P. putida* was grown in BG broth, similar to *R. solanacearum*, at 28°C, whereas LB broth was used for culture of *E. coli* and *B. subtilis* at 37°C in a shaking incubator maintained at 150 rpm. All of the inoculums were prepared following the same procedure used for *R. solanacearum* F1C1 mentioned above.

Root inoculation of *R. solanacearum* in tomato seedlings. Approximately 15 to 20 ml of *R. solanacearum* F1C1 inoculum (approximately 10^9 CFU ml^{-1}) was taken in a sterile container (Fig. 1A and B). From the germinated seedling tray, 6- to 7-day-old tomato seedlings were picked one at a time. Roots of each seedling were then dipped in the bacterial inoculum (up to the root-shoot junction) followed by transfer of the seedling to an empty 1.5- or 2.0-ml sterile microfuge tube. All of the seedlings were inoculated by the same procedure. The root-dip-inoculated seedlings transferred to microfuge tubes were subjected to air exposure for approximately 5 min prior to addition of 1 to 1.5 ml of sterile water to each tube. Exposure of the inoculated roots to air at this step was found to be critical for *R. solanacearum* pathogenicity in tomato seedlings.

In all of the experiments, 40 seedlings at minimum were taken for each bacterial inoculum used. In the control set, 40 seedlings were mock inoculated with sterile distilled water following the same

TABLE 1. Bacterial strains and plasmids used in this study

Sl number	Strain	Characteristics[a]	Reference or source
Ralstonia solanacearum			
1	F1C1	Wild-type virulent *R. solanacearum* strain (phylotype I) 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, derived from F1C1	This work
5	TRS1013	*phcA*::Ω; Spc^r, PhcA deficient, exopolysaccharide deficient, 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 bacteria			
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

[a] Rif^r, Spc^r, Amp^r, and Gen^r indicate resistant to rifampicin, spectinomycin, ampicillin, and gentamycin, respectively. Vir^+ and Vir^- indicate virulence proficiency and virulence deficiency, respectively.

steps as mentioned above. All of the inoculated seedlings along with controls were transferred to a growth chamber maintained at 28°C and 75% RH, with a 12-h photoperiod. Seedlings were analyzed for disease progression from the next day onward till the seventh day postinoculation and findings were recorded.

Seedlings of four commercially available tomato cultivars—namely, Akhilesh (Param Hybrid Seed), 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 nonpathogenic bacteria such as *B. subtilis*, *P. putida*, and *E. coli* in tomato seedlings was also done as above.

Root inoculation of tomato seedlings with different titers of *R. solanacearum* F1C1. Seven-day-old tomato seedlings of Durga (selection -22) were inoculated with different dilutions (10° to 10^6) (i.e., 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 CFU ml^{-1} inoculum) of *R. solanacearum* F1C1 by the root inoculation technique described above to determine the effect of different titers of pathogen on disease progression. Sets of 40 seedlings were recruited in

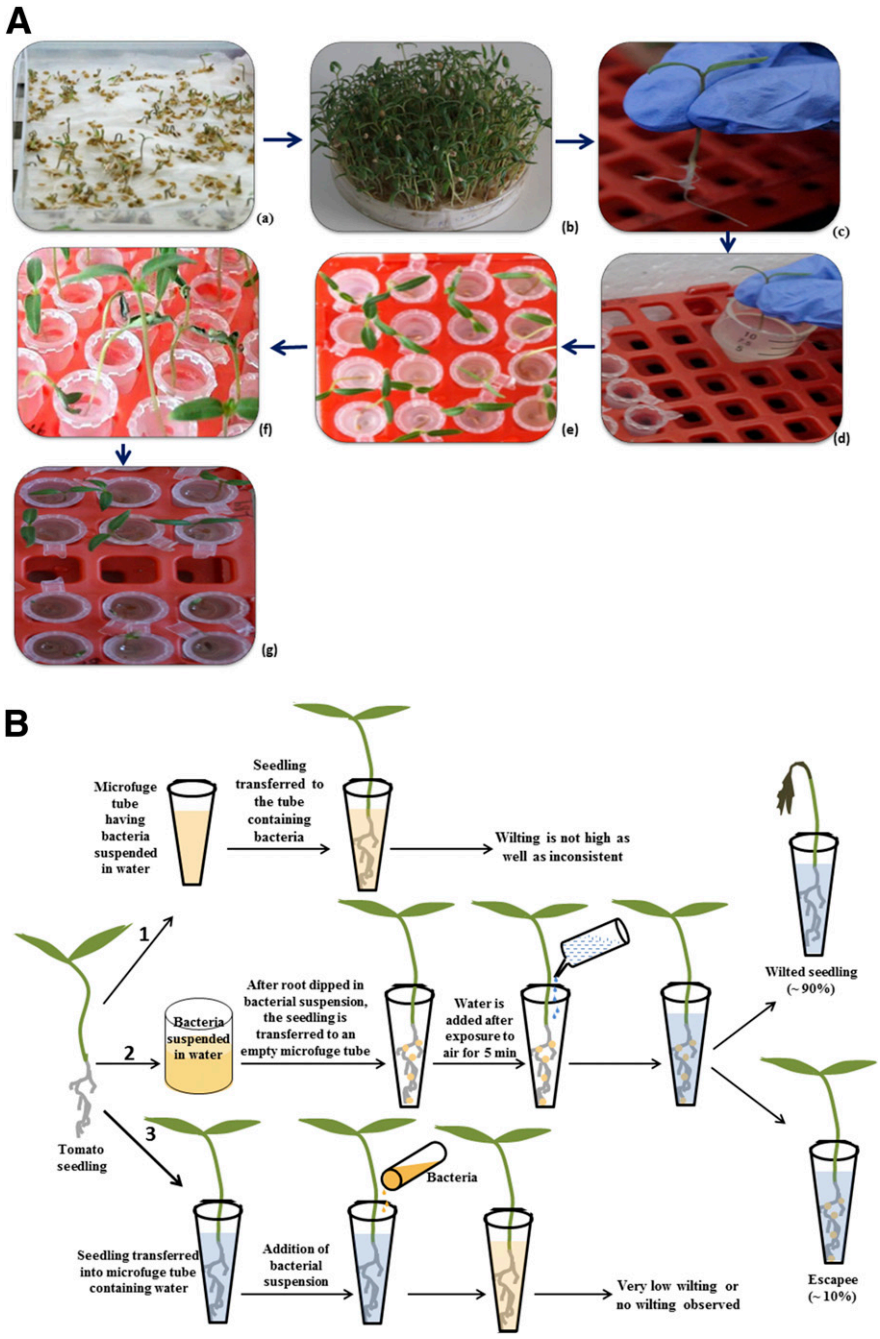

Fig. 1. A, Pictures describing different steps involved the root inoculation method to study *Ralstonia solanacearum* pathogenicity in tomato seedlings. **(a)** Germination of tomato seed on a sterile and wet tissue paper bed. Prior to spreading, seeds were washed and soaked for 24 h. Seeds were allowed to germinate in a growth chamber at 28°C and 75% relative humidity up to the seedling stage. **(b)** Germinated seedlings in a plate after 6 to 7 days. **(c)** A 7-day-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- to 2-ml tubes. After approximately 5 min of exposure to air, 1.0 to 1.5 ml of sterile water was added to the microfuge tube. The tray containing the microfuge tubes having the seedlings was kept inside the growth chamber. **(f)** Within 48 h, infected seedlings started wilting. **(g)** After 7 days, 80 to 90% of infected tomato seedlings were wilted or had died (lower side) in comparison with water control (upper side). **B,** Schematic representation of the *R. solanacearum* pathogenicity in tomato seedlings by the root inoculation method. The 6- to 7-day-old tomato seedlings were root dipped in bacterial suspension and then transferred to microfuge tubes. After approximately 5 min of exposure to air, water was added to the microfuge tube and the tube incubated in the growth chamber. Wilting symptoms started appearing from the second day postinoculation onward. To observe maximum wilting in the seedlings, sequence number 2 is to be followed. In sequences 1 and 3, infection was found to be inconsistent and low.

each dilution inoculation. Bacterial pelleting was performed as stated in previous sections. The resuspended bacterial pellets were serially diluted to obtain *R. solanacearum* (F1C1) at approximately 10^9 to 10^3 CFU ml^{-1} of by adding sterile distilled water prior to inoculation. Seedlings were analyzed for disease progression till the seventh day postinoculation and data were 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 h at 28°C. The bacterial inoculum (50 µl) was mixed with 1 to 2 µg of linearized genomic DNA as well as plasmid DNA and spotted on 0.45-µm sterile nitrocellulose membrane kept over a BG agar plate without glucose. After 48 h of incubation at 28°C, 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 *Apa*I and naturally transformed into *R. solanacearum* F1C1. mCherry-labeled transformants were selected on BG agar plates containing gentamycin antibiotic. One of the mCherry-labeled strains (TRS1016) was cultured in BG broth containing an appropriate antibiotic. TRS1016 culture was pelleted down and inoculum of the former at 10^9 CFU/ml was prepared by the same method described for *R. solanacearum* above. TRS1016 inoculum was used for root inoculation of 7-day-old tomato seedlings, as stated above. After 3 days postinoculation, tomato seedlings were surface sterilized following the method of Kumar et al. (2017). Sterilized seedlings were observed under the fluorescence microscopy (EVOS FL; Life Technologies) at ×40 magnification adjusted in a red fluorescent protein filter.

Creation of *pilT and rpoN2* mutants of *R. solanacearum* F1C1. Taking the reference genome of GMI1000 from the LIPM database (https://iant.toulouse.inra.fr/), primers were designed for partial amplification of *rpoN2* and *pilT* gene homologs in the *R. solanacearum* F1C1 strain. In all of the sets of primers designed, forward primers contained a *Hin*dIII restriction site and the reverse primers were incorporated with an *Xba*I restriction site at their 5′ ends. Primers sequences are given in Supplementary Table S8. Primers (5′-GCCAAGCTTGCTGCCAAGAACAAAGCGTCT-3′ and 5′-GCCTCTAGATCCCGCAGCGCCGATT-3′) were used for amplification of an approximately 500-bp amplicon of the *pilT* gene homolog in F1C1. This amplicon was sequenced for confirming homology with the *pilT* as well as *rpoN2* sequences of the GMI1000 strain. The amplicon was ligated to a T-A cloning vector pTZ57R/T (Thermo Fisher Scientific) to get a construct pNST1 (pTZ57R/T::$pilT_{F1C1}$) following the manufacturing company's instructions. pNST1was subjected to restriction digestion with *Hin*dIII and *Xba*I enzymes simultaneously. The resulting approximately 500-bp amplicon from the previous step was gel extracted and, subsequently, ligated to the pCZ367 (Cunnac et al. 2004) vector that harbors a promoterless *lacZ* reporter gene and ampicillin and gentamycin selection markers. Prior to ligation setup, 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 the cloning step with digestion of pNST001 with *Hin*dIII and *Xba*I enzymes.

Primers (5′-GCCAAGCTTGCGACCGAATTTGCACAGG-3′ and 5′-GCCTCTAGACGTCTTCGGCCTCGATCAT-3′) were used to amplify an approximately 1.3-kb amplicon of *rpoN2* homolog in F1C1. This amplicon was confirmed for homology with the *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 the 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 5-bromo-4-chloro-3-indolyl–β-D-galactoside activity. Insertion mutations in *pilT* and *rpoN2* genes of successful transformants were confirmed by polymerase chain reaction. The phenotype of the *pilT* mutant was also studied for twitching motility deficiency (Supplementary Fig. S7), following the 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* mutants of *R. solanacearum* F1C1. To create *hrpB* and *phcA* mutations in the 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 the 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 a previous step—TRS1012 (*hrpB* mutant F1C1) and TRS1013 (*phcA* mutant F1C1)—were recruited in subsequent studies. *hrpB* mutants of F1C1 were found to be deficient to elicit the hypersensitive response in tobacco leaves after infiltration, as well as virulence deficient in tomato seedlings by root inoculation, unlike the wild-type F1C1 (Fig. 2). The *phcA* mutant colonies were transparent due to being deficient for exopolysaccharide production, exhibited very high motility on semisolid agar medium, and were virulence deficient, unlike the wild-type F1C1 (Fig. 2).

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- to 7-day-old tomato seedlings under gnotobiotic root inoculation of *R. solanacearum* confirmed the susceptibility of early stages of tomato seedlings toward the pathogen (Kumar 2014). In this setup, each tomato seedling (S22 Evergreen variety) kept in a 1.5-ml 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 the 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.

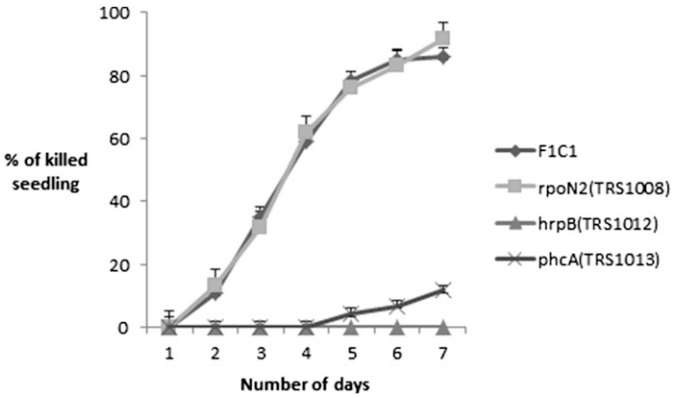

Fig. 2. Virulence data of F1C1, *hrpB*, *phcA*, and *rpoN2* strains in the root inoculated tomato seedlings. The x-axis represents the days postinoculation and y-axis represents the percentage of wilted seedlings. It is distinctly clear that the *hrpB* mutant is nonpathogenic whereas the *phcA* mutant is significantly reduced for virulence, and the *rpoN2* strain is like the F1C1 wild type.

Although our efforts to find an effective root inoculation method in 6- to 7-day-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). First, 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 at 5 min, although an instant air exposure was also sufficient to result in virulence of the tomato seedlings. Second, direct addition of *R. solanacearum* suspension to roots of tomato seedlings already submerged in water resulted in lesser or inconsistent disease symptoms. Another method (i.e., directly transferring the seedlings into a microfuge tube already containing *R. solanacearum* suspension) also did not result in very high numbers of wilting symptoms of the seedlings. Disease progression as well as pathogenicity due to *R. solanacearum* were 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 h postinoculation. In fact, observation of pathogenicity symptoms by the root inoculation method is faster than the leaf clipping inoculation method, in which the disease symptoms appeared on the third day postinoculation (Kumar et al. 2017). On the seventh day postinoculation via this root inoculation method, approximately 80 to 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. solanacearum* inoculation, seedlings were also inoculated with few nonpathogenic bacteria such as *P. putida, B. subtilis*, and *E. coli*. After the seventh day postinoculation, none of the seedlings inoculated with these nonpathogenic bacteria exhibited disease symptom (Supplementary Fig. S1; Supplementary Table S1). This outcome indicated that death of the seedlings occurred due to *R. solanacearum* F1C1 via this mode of inoculation. Further *R. solanacearum* F1C1 pathogenicity of similar magnitude could be observed in three other tomato cultivars: Durga (Ruby), Akhilesh, and Vijay (Supplementary Fig. S2; Supplementary Table S2). We further studied F1C1 pathogenicity in tomato seedlings by root inoculation as a function of the pathogen concentration in the inoculum. Bacterial concentration varied from approximately 10^9 to 10^3 CFU ml^{-1}. Disease symptoms were observed distinctly up to approximately 10^5 CFU/ml. It is also observed that the pathogenicity and disease progression magnitude decreased as bacterial concentration in the inoculum decreased (Supplementary Fig. S3; Supplementary Table S3).

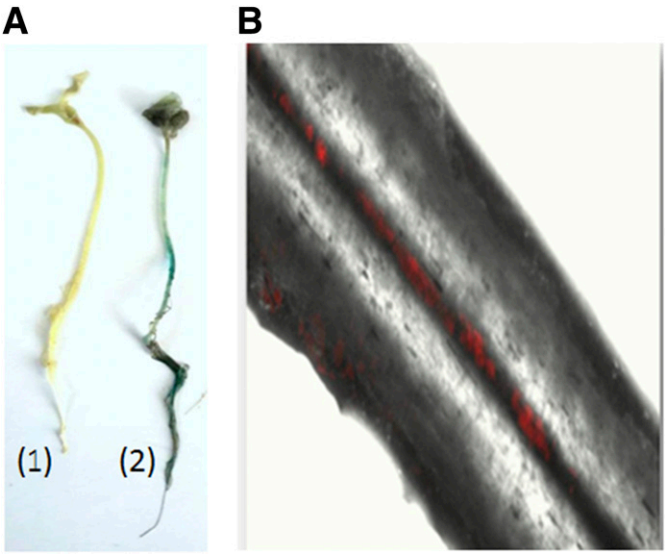

Fig. 3. A, X-gluc staining of the seedlings root inoculated with *Ralstonia solanacearum* marked with *gus*. In this picture, the staining of seedling 2 suggests that it is *gus* positive. For the *gus* assay, this tomato seedling was root inoculated with a *gus*-marked *Ralstonia solanacearum* strain (TRS1002). The staining in the root and shoot regions confirmed the bacterial colonization in the pathogen-infected seedling while other tomato seedlings (such as seedling 1) were control seedlings, where no bacteria were inoculated; these appeared as *gus* negative. **B,** Tracking the presence of *R. solanacearum* in tomato seedlings inoculated with mCherry-labeled bacteria. For further confirmation of pathogen colonization inside infected tomato seedlings, we inoculated an m-Cherry-tagged *R. solanacearum* strain (TRS1016) in tomato seedlings through the root. In this image, fluorescence was observed in the stem region, confirming the presence and colonization of m-cherry-tagged *R. solanacearum* inside the tomato seedling.

The association of F1C1 with tomato seedling inoculated by this method was studied further by using *R. solanacearum* F1C1-derived strain TRS1002 (*gus* marked) as well as TRS1016 (mCherry marked) to study bacterial colonization in the seedlings. Bacterial colonization in the infected seedlings was observed from root to the shoot regions (Fig. 3A and B). This suggested that, after the inoculation, the bacterium migrated from root to shoot regions during the infection process and resulted in pathogenesis.

The root inoculation method can be used to study *R. solanacearum* virulence functions. To further evaluate that this root inoculation method is useful in studying virulence functions of *R. solanacearum*, we inoculated tomato seedlings with different *R. solanacearum* mutants such as *hrpB* (TRS1012), *phcA* (TRS1013), and *rpoN2* (TRS1015) (Table 1). As anticipated, the *hrpB* mutant was nonpathogenic, the *phcA* mutants was found to be significantly reduced for virulence, and the *rpoN2* mutant exhibited virulence proficiency (Fig. 2; Supplementary Fig. S4; Supplementary Table S4). The virulence phenotypes of *hrpB, phcA*, and *rpoN2* mutants were in concordance with the virulence phenotype data reported earlier in adult tomato plants (Ray et al. 2015). A characteristic yellowish color of the cotyledon leaves was observed in the case of seedlings inoculated with the *phcA* mutant. However, a leaf-clip-inoculated *phcA* mutant 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 (Kang et al. 2002; Liu et al. 2001). We created a *pilT* mutant of F1C1 in this study (TRS1014), which was deficient for twitching motility. TRS1014 was found to be moderately virulence deficient in tomato seedlings, unlike *hrpB* and *phcA* (Fig. 4; Supplementary Fig. S5; Supplementary Table S5). This proved that the root inoculation method in tomato seedlings is useful to discriminate between severely and moderately virulence-deficient strains of *R. solanacearum*. Interestingly, TRS1014 was further found to be virulence proficient by the leaf-clip inoculation method in tomato seedlings (Supplementary Fig. S6; Supplementary Table S6). This might be due to differential requirements of twitching motility during the two modes of infection.

DISCUSSION

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*, *phcA*, and *pilT*. Through this inoculation process, *R. solanacearum* strains tagged with *gus* and mCherry were recruited for observing bacterial colonization in tomato seedlings which manifested colonization of the bacteria in root as well as shoot regions. The method is reasonably simple, easy, and rapid. Because this involves less inoculum of bacteria as well as less space, large-scale screening of tomato seedlings for an *R. solanacearum* virulence assay is possible by this method. It is pertinent to note that, previously, root inoculation of this pathogen has been performed in plantlets of *Medicago truncatula* as well as tomato by germinating seed under sterile conditions in the presence

or absence of nutrients (Vailleau et al. 2007; Vasse et al. 1995). Because of the involvement of tissue-culture-based techniques, these methods will not be easy for doing pathogenicity study in large scale. Recently, Lu et al. (2017) reported *R. solanacearum* root infection in early stages of *A. thaliana* under gnotobiotic condition.

The extensive pathogenicity of *R. solanacearum* in 6- to 7-day-old tomato seedlings within 48 h of root inoculation is a demonstration of its virulence in very early stages of plant growth. Though similar pathogenicity in tomato seedlings was reported earlier by the same authors using leaf-clipping inoculation, the pathogenicity in seedlings by root inoculation holds much more significance than the leaf-clipping inoculation because the pathogen enters the host plant through its roots during natural infection. An interesting point in regard to leaf clipping, where disease symptoms in tomato seedlings were noticed to begin at the point of inoculation and then progress downward (Kumar et al. 2017), is that, through this root inoculation process, disease appearance could be observed in the shoot region without any visible sign of effects at the root region. Furthermore, the characteristic bending of the upper shoot region in the beginning of the disease in seedlings was observed by root inoculation but was not observed in seedlings by leaf-clipping inoculation. 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 the *pilT* mutant through the root inoculation method but not by the leaf-clipping inoculation 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 the *R. solanacearum* entry mechanism through the root might be more 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 method of root inoculation by soil drenching, whereas they were virulence proficient when inoculated by the 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 the initial population of bacteria during infection and their growth inside the seedlings are important for the disease symptoms. How the pathogen population affects the disease in plants in the case of *R. solanacearum*, which is a systemic pathogen in comparison with other bacterial pathogens that are tissue specific, is an interesting question for future research.

In the method presented here, immediate exposure of roots 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 roots in our experiment was set for 5 min (Fig. 1A and B), although even an instantaneous exposure to air is sufficient to cause pathogenicity symptoms in tomato seedlings. Although the precise role of air exposure is unknown, claims of Yao and Allen (2007) regarding involvement of aerotaxis in *R. solanacearum*–tomato plant interaction might indicate involvement of an identical mechanism during the pathogen–tomato seedling impingement. The authors (Yao and Allen 2007) tested *R. solanacearum* root colonization by incubating tomato seedlings in bacterial inoculums for 30 min, although there was no mention of virulence in them. A probable role of air exposure or aerotaxis also may be predicted in the root-dip assay conducted by Park et al. (2007) in 6-week-old tomato plants, where they incubated tomato plant roots in bacterial suspension for an equal duration as in the case of Yao and Allen (2007) before transferring the plants to soil. Similarly, in their pathogenicity assay, Maji and Chakrabartty (2014) immersed surgically wounded roots of tomato plantlets in an *R. solanacearum* suspension for 3 h. Unknowingly, the transient period between transfers of the plants from bacterial suspension to the respective culture conditions in their experiments might have played a role during infection progression. In addition to the above, during our infection study, we observed an interesting infection behavior of *R. solanacearum* in the case of prewet tomato seedlings. When tomato seedlings were incubated for 24 h in a microfuge tube with the root region submerged in sterile water, then inoculated with *R. solanacearum* by the root inoculation method, the seedlings were surprisingly found to be less susceptible to wilting symptoms (Supplementary Fig. S8). However, bacterial colonization was observed inside the inoculated prewet tomato seedlings. It is interesting to note that the tomato seedlings under the same prewet conditions were susceptible to *R. solanacearum* pathogenicity when inoculated by the leaf-clipping method. Whether *R. solanacearum* is deficient in its attachment to the roots of the prewet tomato seedlings will require further investigation.

Considering the potential benefits of the root inoculation method described here, we anticipate that this inoculation method will help the world scientific community to address several fundamental questions pertaining to *R. solanacearum* interaction with seedling stages of the host and aid in foreseeing mechanisms of virulence in adult plants. This method might turn out to be instrumental in devising suitable biocontrol measures against the wilt pathogen in the immediate future.

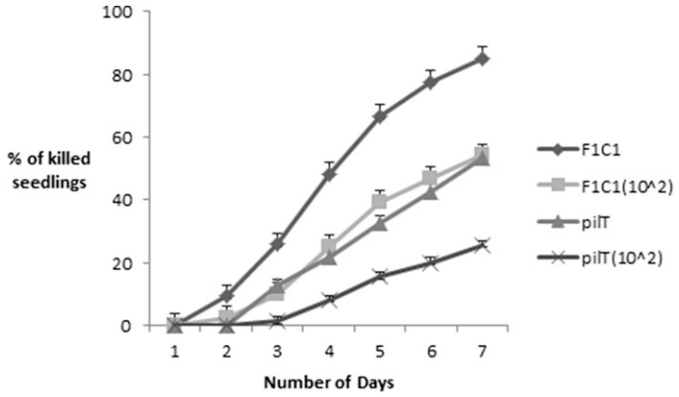

Fig. 4. *pilT* is virulence deficient by root inoculation. The x-axis represents the days postinoculation and the y-axis represents the percentage of wilted seedlings. It is distinctly clear that the *pilT* mutant is reduced for virulence by root inoculation in comparison with the F1C1 wild type. We compared the F1C1 wild type with the *pilT* mutant in two different concentrations of bacteria in the inoculum (100-fold dilution and 102-fold dilution). In both concentrations, *pilT* was found to be reduced for virulence.

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