Study of virulence factors and identification of effector proteins in *Ralstonia solanacearum*

Submitted to

GOA UNIVERSITY

For the award of the degree of

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

by

Trupti Asolkar

Reg. No. 201210117

Department of Microbiology, Goa University

Guide

Dr. R. Ramesh

Principal Scientist, Plant Pathology,

ICAR-Central Coastal Agricultural Research Institute, Goa.

Work carried out at ICAR-CCARI, Goa

August 2018

CERTIFICATE

This is to certify that the work presented in the accompanying thesis entitled "**Study of virulence factors and identification of effector proteins in *Ralstonia solanacearum***", has been carried out by **Ms. Trupti Asolkar** under my guidance and supervision in the Plant Pathology Laboratory, ICAR- Central Coastal Agricultural Research Institute, Ela, Old Goa, Goa.

This thesis has been submitted for the award of degree of **Doctor of Philosophy in Microbiology**, to the **Goa University**, Taleigao Plateau, Goa. The work carried out by the candidate is original and has not been submitted to any other Institute or University.

Research Guide
R. Ramesh
Principal Scientist,
ICAR-CCARI

DECLARATION

I hereby certify that this thesis entitled **"Study of virulence factors and identification of effector proteins in *Ralstonia solanacearum*"** submitted for the award of degree of **Doctor of Philosophy in Microbiology**, to the **Goa University** is entirely my original work and neither any part of this thesis nor the whole thesis has been submitted for a degree to any other University or Institution. The material and information used or derived from other published or unpublished sources has been clearly cited and appropriately acknowledged.

As suggested by the external examiners, appropriate corrections are incorporated in the relevant pages of this thesis.

Trupti Asolkar
Department of Microbiology
Goa University

ACKNOWLEDGEMENT

Working as a Ph.D. student in ICAR-Central Coastal Agricultural Research Institute, Ela, Old Goa, Goa, was splendid as well as a challenging experience for me. During the coarse of my academic career, many people were instrumental either directly or indirectly in shaping my success. It is next to impossible for me to prosper in my doctorial work without the support of these personalities. Here is a small tribute to all those people.

First of all, I wish to thank my research guide and supervisor, Dr. R. Ramesh, Principal Scientist, ICAR-CCARI. It was due to his valuable guidance, enthusiasm and support that I was allowed to explore on my own. He guided me with my topic of research, its objectives, difficulties and various methodologies to achieve my results. I'm thankful to him for allowing me to use laboratory and other facilities during the coarse and also for his great help especially during the final years of my research, such as thesis correction, etc. I feel fortunate to have a guide like him who not only raised standards of my knowledge enrichment but also helped and most importantly believed in me to achieve those standards.

I wish to thank Dr. E.B. Chakurkar, Director, ICAR-CCARI and former Director, Dr. N. P. Singh, for their permission to use facilities for my research work at ICAR-CCARI. My research work was impossible without financial aid by ICAR, New Delhi, India. Their help in the form of Senior Research Fellowship through "Outreach project on *Phytophthora, Fusarium* and *Ralstonia* diseases of horticultural and field crops-(*PhytoFuRa*)" was of great help.

I also wish to thank the Faculty Research Committee members, Dr. M.K. Janarthanam, Dean, Goa University, Dr. N.L. Thakur, Vice Chancellor's nominee, Dr. Sandeep Garg, Head of Department, Department of Microbiology and Dr. S. Nazareth for their valuable suggestions while reviewing my research progress. Also special thanks to Dr. S.K. Dubey for his suggestions and permission to use laboratory facilities at Department of Microbiology, Goa University.

I am grateful to Dr. Marc Valls, Associate Professor in Genetics, Department of Genetics, University of Barcelona, for providing the pRCG-Pep-GWY destination vector and the protocol for validation of T3E.

My humble gratitude to Dr. Chethan Kumar H.B. Scientist, Veterinary Public Health, ICAR-CCARI, for the guidance and laboratory facilities during my RNA work. I also thank Dr. A.R. Desai, Principal Scientist, Fruit Science, for his motivation and support.

I wish to thank Ms. Shweta Naik and Ms. Marsha D'souza for their help and support at ICAR-CCARI. Also my colleagues and supporting staff Mrs. Prafulla Khandeparkar, Mrs. Shanti Gaonkar, Ms. Gauri Achari, Mr Sitaram Kuncolikar, Mr. Siddesh Menon, Ms. Neelima Dassari and Ms. Tulsi Gaonkar for their valuable contribution. I also thank Ms. Jaya Sharma and Mr. Kashif Shamim, Department of Microbiology for their help and co-operation.

My gratitude will be incomplete without the mention of my biggest support, my family, Mr. Shyamsundar Asolkar, Mrs. Kiran Asolkar, Mrs. Preeti Asolkar and Mr. Sushil Pandita. Also my other family members and friends who are equally responsible for my success.

Finally I would like to thank the almighty and those people whom I could not mention but they were there by my side giving strength to achieve my goals.

Trupti Asolkar

ABBREVIATIONS

RSSC	*Ralstonia solanacearum* species complex
EPS	Extracellular polysaccaharides
T3SS	Type III secretion system
T3E	Type three effector
hrp	hypersensitive response and pathogenicity
T2SS	Type II secretion system
PCWDE	Plant cell wall degrading enzyme
T6SS	Type VI secretion system
OD	Optical density
TZC	Tetrazolium chloride
PCR	Polymerase chain reaction
CFU	Colony forming units
HA	Hemagglutinin
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
DAI	Days after inoculation
DPI	Days post inoculation
Cv	Cultivar
var	Variety
rpm	Revolutions per minute
RT	Room temperature
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
IPTG	Isopropyl β-D-1-thiogalactopyranoside
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
TAE buffer	Tris Acetate EDTA buffer
APS	Ammonium per sulphate
TEMED	Tetramethylenediamine

UNITS OF MEASUREMENT

M	Micro
G	Grams
Mg	Microgram
Ng	Nanograms
mg	Miligrams
µL	Microlitre
mL	Milliliter
M	Molar
mM	Milimolar
µM	Micromolar
Nm	Nanometer
$CFU.g^{-1}$	Colony forming units per gram
$CFU.mL^{-1}$	Colony forming units per mL
$\mu g.mL^{-1}$	Microgram per milliliter
rpm	Revolution per minute
^{o}C	Degree Celsius
U	Units
V	Volts
kV	Kilovolts
Bp	Base pairs
Kb	Kilobase
S	Seconds
min	Minutes
H	Hour
kDa	Kilodaltons

LIST OF FIGURES

Fig. No.	Description	Page no.
2.1.	Bacterial wilt caused by *R. solanacearum* in brinjal	5
2.2.	*R. solanacearum* colonies on CPG/BG medium	6
2.3	Classification of *R. solanacearum*into three species	12
3.1	Phenotypic depiction of virulence factors present in *R. solanacearum* isolate Rs-09-161	42
3.2	Venn diagram constructed using T3Es complete genes of *R. solanacearum* isolates Rs-09-161, Rs-10-244 and GMI1000.	55
3.3	Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipA (AWR family) of *R. solanacearum*isolates.	58
3.4	Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipG (GALA family) of *R. solanacearum*isolates.	59
3.5	Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipH (HLK family) of *R. solanacearum*isolates.	60
3.6	Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipS (SKWP family) of *R. solanacearum*isolates.	61
4.1	Vector map of linearized vector pTZ57R/T	71
4.2	Region of insertion of internal fragment of the gene with ddA overhang in the pTZ57R/T vector sequence.	73
4.3	Diagrammatic representation of mutagenesis of gene by homologous recombination.	78
4.4	Typical *R. solanacearum* colonies on BG media supplemented with TZC	79
4.5.	Amplification of internal fragments of the genes selected for mutagenesis	81
4.6	Colony PCR for the confirmation of the selected T3SS and T6SS gene insert in *E.coli* colonies.	83

Fig. No.	Description	Page no.
4.7	Restriction digestion of the recombined plasmids containing the internal fragment of selected genes.	84
4.8	Confirmation of insertional mutagenesis in *R. solanacearum* Rs-09-161.	86
4.9	Phenotype of wild type Rs-09-161 and T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$.	88
5.1	Bacterial wilt incidence in brinjal (*Agassaim*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T3SS mutants: Rs-HrpB$^-$ and Rs-HrcV$^-$, by soil drench inoculation	100
5.2	Bacterial wilt incidence in tomato (*Pusa Ruby*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$ on tomato by soil drench inoculation.	101
5.3	Bacterial wilt incidence in brinjal (*Agassaim*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T3SS mutants: Rs-HrpB$^-$ and Rs-HrcV$^-$, by cut petiole inoculation	103
5.4	Bacterial wilt incidence in tomato (*Pusa Ruby*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T3SS mutants on tomato by cut petiole inoculation.	104-105
5.5	In plants assay using *R. solanacearum* isolates Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$	106
5.6	Bacterial wilt incidence in tomato (*Arka Vikash*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T6SS mutants by soil drench inoculation	110
5.7	Bacterial wilt incidence in brinjal (cv., *Agassaim*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T6SS mutants by soil drench inoculation	113
5.8	Bacterial wilt incidence in tomato (*Arka Vikash*) inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T6SS mutants on tomato by cut petiole inoculation	116-117
5.9	Bacterial wilt incidence in brinjal (cv., *Agassaim)* inoculated with *R. solanacearum*wild type isolate Rs-09-161 and its T6SS mutants by cut petiole inoculation	120-121

Fig. No.	Description	Page no.
5.11	*In planta* assays with *Arka Vikash* using *R. solanacearum* isolates Rs-09-161 and its T6SS mutants	127
5.12	Interbacterial competition assay	133
6.1	Vector map of pENTR-SD-D-TOPO entry vector	141
6.2	Backbone of pRCG vector series	146
6.3	Amplification of T3E genes, *RipAM* and *RS15E*	157
6.4	Colony PCR for the confirmation of *RipAM* and *RS15E* in the pENTR-SD-D-TOPO vector	158
6.5	Confirmation of RipAM and RS15E in pRCG-Pep-RipAM and pRCG-Pep-RS15E after LR clonase reaction	160
6.6	Confirmation of pRCG-Pep-RipAM and pRCG-Pep-RS15E in wild type *R. solanacearum* and its T3SS mutants	161
6.7	Validation of T3E RS15E in the cell supernantant through Western blotting	163
6.8	Amplification of *RipAM* gene from cDNA in wild type Rs-RipAM and its T3SS mutant Rs-hrpB-RipAM	164

LIST OF TABLES

Table No.	Description	Page no.
2.1.	Biovar classification of *R. solanacearum*	9
2.2.	Differentiation of *R. solanacearum* strains into races based on the host range and geographical distribution	10
3.1	*R. solanacearum* strains used in this study	35
3.2.	Identification of various virulence factors in *R. solanacearum*	37-39
3.3	Comparison of the General features of the *R. solanacearum* strain Rs-09-161 and Rs-10-244 with GMI1000	43
3.4	Sequence similarity of genes coding for EPS of Rs-09-161 and Rs-10-244 with representative phylotype strains	46
3.5	Sequence similarity of genes coding for PCWDE of Rs-09-161 and Rs-10-244 with representative phylotype strains	46
3.6	Sequence similarity of genes coding for chemotaxis, swimming motility and twitching motility of Rs-09-161 and Rs-10-244 with representative phylotype strains	47
3.7	Identification of T3Es in *R. solanacearum* strains Rs-09-161 and Rs-10-244	49-54
4.1	List of genes used in the study, details about the locus tag, size and location	66
4.2	List of primers used for the amplification of internal fragment of genes	68
4.3	Reaction mixture and PCR conditions for amplification of internal fragment of gene	69
4.4	Ligation mixture for cloning pTZ57R/T vector	70
4.5	Concentration of insert fragment (0.52 pmol ends) used in ligation	71
4.6	Restriction profile of the recombined vectors used for mutagenesis in *R. solanacearum*	74
4.7	List of diagnostic primers used in the study	76

Table No.	Description	Page no.
4.8	Details about the reaction mixture and PCR conditions for confirmation of mutagenesis of gene in *R. solanacearum*	77
4.9	List of plasmids and strains used in the study	80
4.10	Population of *R. solanacearum* mutant Rs-hrpB$^-$ and Rs-hrcV$^-$ in the absence of antibiotic selection to study the stability	87
5.1	*R. solanacearum* mutants used in the virulence assay	92
5.2	Number of experiments in virulence assays of T3SS mutants	94
5.3	Number of experiments in virulence assays of T6SS mutants	96
5.4	Plant colonisation by *R. solanacearum* strains Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$	108
5.5	Wilt incidence caused by the T6SS mutants on susceptible tomato (var., *Arka Vikash*) by soil drench inoculation	111
5.6	Wilt incidence caused by the T6SS mutants$^-$ in susceptible brinjal (cv., *Agassaim*) by soil drench inoculation	114
5.7	Wilt incidence caused by the T6SS mutants in susceptible in tomato (var., *Arka Vikash*) by cut petiole inoculation	118
5.8	Wilt incidence caused by the T6SS mutants in susceptible in brinjal (cv., *Agassaim)* by cut petiole inoculation	122
5.9	Wilt incidence caused by the T6SS mutants in moderately resistant variety of tomato (*Arka Rakshak*) by cut petiole inoculation	126
5.10	Details about the number of days required to cause 50% and 75% wilt incidence in the inoculated seedlings	128
6.1	List of genes used in the study, details about the locus tag, size and location in Rs-09-161	137
6.2	List of plasmids and strains used in this study	138
6.3	List of primers used for the amplification of T3E genes	139

Table No.	Description	Page no.
6.4	The reaction mixture and PCR conditions for amplification of T3E gene	139
6.5	Ligation reaction for cloning of effector gene in pENTR-SD-D-TOPO vector	140
6.6	Sequence of primers used for the confirmation of T3E gene in recombinant entry vector	142
6.7	Reaction mixture and PCR conditions for confirmation of T3E gene in recombinant entry vector	143
6.8	Restriction profile of the recombined entry vectors	144
6.9	Sequences of primers used in the confirmation of effector construct in *R. solanacearum*	149
6.10	Reaction mixture and PCR conditions for confirmation of effector construct in *R. solanacearum*	149
6.11	Primary and secondary antibody used in this study	154

INDEX

Chapter no.	Name	Page no.
I	Introduction	1-3
II	Review of literature	4-32
III	Prediction of probable virulence factors, effector proteins in *Ralstonia solanacearum* through genomic analysis.	33-62
IV	Development of mutants of *R. solanacearum* lacking important virulence factors.	63-89
V	Virulence determination of mutants through functional assays or plant assays.	90-134
VI	Validation of effector protein secretion through Type Three Secretion System.	135-165
VII	Summary	166-167
VIII	Bibliography	168-188
IX	Appendix	
	Appendix I: Virulence assays	189
	Appendix II: Media composition	190-191
	Appendix III: Buffers and reagents	192-194
	Appendix IV: Accession numbers	195
	Appendix V: Strains used in the study	196-197
X	Publications	198

CHAPTER I

Introduction

Ralstonia solanacearum is a devastating, soil-borne plant pathogenic bacterium, which causes severe wilt in crop plants (Genin and Denny, 2012). It has a worldwide distribution and an unusually wide host range of 450 plant species which belong to more than 54 botanical families (Wicker *et al.*, 2007). The host plants include solanaceous crops *viz*. potato, tomato, tobacco, chilli, sweet pepper, brinjal; as well as among non solanaceous crops like banana, geranium, ginger, olive, groundnut, bean, sunflower; marigold, custard apple, cowpea, cashew and many other plants including ornamental plants (Alvarez *et al.*, 2010). It has been ranked second in the list of top 10 of the most studied bacterial plant pathogens in molecular plant pathology field (Mansfield *et al.*, 2012). This pathogen causes huge economical loss in agriculturally important crops due to its destructive nature, lethality and persistence (Wicker *et al.*, 2004). For instance, yield loses were estimated to be in the range of 10-100% in potato and peanuts depending upon the crop seasons in China (Chen *et al.*, 2005; Yu *et al.*, 2011). *R. solanacearum* strains have been isolated from a large number of plants globally and the diversity among the strains was analysed using various methods. This has led to classify this group of organisms into "*Ralstonia solanacearum* species complex (RSSC)" (Fegan and Prior, 2005).

R. solanacearum normally enters the host through a wound, mostly through the roots. The bacterium gets access to the wounds by chemotaxic attraction towards root exudates and flagellar-mediated swimming. Once entered into a susceptible host plant, it colonises in the root cortex to form sufficient cell density. In the roots, the xylem vessels are invaded which helps in the spread of the bacterium in a systemic manner along the plant causing vascular dysfunctioning. This bacterium can survive in moist soils for years (Genin and Denny, 2012).

The ability of *R. solanacearum* to cause wilt in plants is attributed to the presence of various virulence factors. These virulence factors include the chemotaxis, flagella driven swimming motility, pili associated twitching motility, the extracellular polysaccharide (EPS), the Type Two Secretory System (T2SS) dependent cell wall degrading enzymes and the Type III Secretory system (T3SS) (Schell, 2000; Saile *et al.*, 1997). These factors play important roles in locating, attaching and colonizing the plants. The mutants of T2SS, chemotaxis, swimming motility and twitching motility displayed reduced virulence and mutants of EPS and T3SS are non-pathogenic (Saile *et al.*, 1997; Tans-Kersten *et al.*, 2001; Meng *et al.*, 2011; Brito *et al.*, 2002). In addition to T2SS and T3SS, other secretion

systems *viz.* T1SS, T4SS, T5SS and T6SS are also present in Gram negative pathogenic bacteria (Green *et al.*, 2016). The T1SS, T2SS and T5SS secrete proteins across the bacterial envelop to the extracellular milieu. The T3SS, T4SS and T6SS deliver proteins directly across host membranes. The Type Six Secretion System (T6SS) has been recently identified in *R. solanacearum* (Zhang *et al.*, 2012), which is known to be involved in inter-bacterial interaction, biofilm formation and eukaryotic cell interaction. (Lossi *et al.*, 2012).

The T3SS encoded by the *hrp* (hypersensitive response and pathogenicity) regulon (Boucher *et al.*, 1987) plays a crucial role in the pathogenicity of *R. solanacearum* (Alfano and Collmer, 2004). The T3SS injects pathogenicity proteins termed as Type III effectors (T3Es) into the eukaryotic hosts through the *hrp* pili (Van Gijsegem *et al.*, 2000; 2002) and are directly translocated into the cytosol of the hosts (Cunnac *et al.*, 2004b; Mukaihara and Tamura, 2009). Once within the host cells, effectors promote the colonisation of the pathogen by interacting with various host proteins and subverting the host immunity (Poueymiro *et al.*, 2014). Whole genome sequencing of *R. solanacearum* strains have revealed the presence of large number of effectors distributed throughout the genome of the bacteria. Many of the T3Es expressed by *R. solanacearum* are validated through translocation studies in GMI1000 and RS1000 with the help of T3SS mutants (Mukaihara and Tamura, 2009; Mukaihara *et al.*, 2010; Sole *et al.*, 2012).

In India, *R. solanacearum* has been isolated from various agriculturally important crops like ginger, (Kumar *et al.*, 2014) potato, (Sagar *et al.*, 2014) tomato, brinjal, chilli (Ramesh *et al.*, 2014a; Kumar *et al.*, 2017) capsicum (Chandrashekara *et al.*, 2012) etc. The list of host plants is continuously increasing including cluster beans, elephant foot yam, water melon, banana, Jute, tobacco, cardamom, coleus, davana, marigold and sunflower (Bholanath *et al.*, 2014; Chandrashekara *et al.*, 2010; Kumar *et al.*, 2012). The severity of the pathogen is widely reported from varying agro-climatic and geographical regions of the country, including the eastern, north east region, western and southern coastal states. Survey conducted in West Bengal during 2004-2007 revealed bacterial wilt in the range of 60-80% in economically important crops like chilli, marigold, brinjal, tomato and ginger (Bholanath *et al.*, 2014). The genetic diversity among the isolated strains is analysed by REP PCR, ITS-PCR, PCR RFLP, MLST and sequencing of *egl, pga* and *hrp* gene (Kumar *et al.*, 2014; Ramesh *et al.*, 2014a). The genome sequence of two *R. solanacearum* strains *viz.* Rs-09-161 and Rs-10-244 infecting solanaceous vegetables isolated from India has been published (Ramesh *et al.*, 2014a; 2014b). Though the genetic

diversity of Indian *R. solanacearum* is reported, the T3SS and T6SS are not studied in these strains. Majority of the pathogenicity and virulence of *R. solanacearum* research has been carried out using the strains, GMI1000 and RS1000.

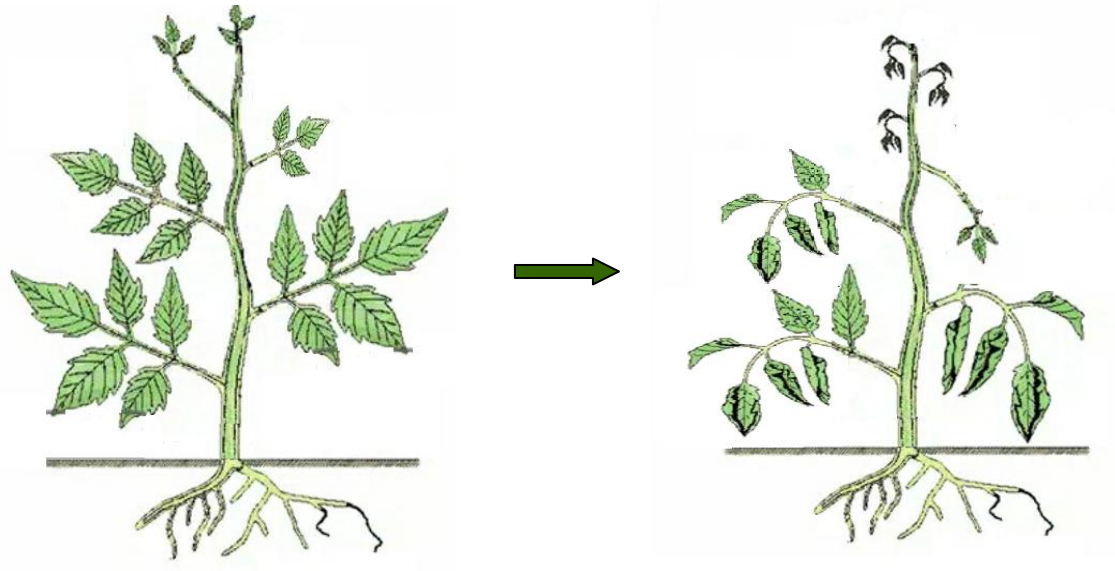
In the coastal region of India, bacterial wilt is severe in solanaceous vegetables and the success in disease management is very limited. Identification and study of additional effectors or new virulence factors will add to the knowledge and help in designing bacterial wilt management strategy. The present research aims at analysing pathogenicity, virulence genes in the Indian strains of *R. solanacearum* and to identify the probable T3Es using bioinformatics approach. It also aims at creating mutants of the T3SS to validate T3Es and mutants of T6SS to study their role in virulence in brinjal and tomato.

The following are the objectives of the study:

1. Prediction of probable virulence factors, effector proteins in *Ralstonia solanacearum* through genomic analysis.
2. Development of mutants of *R. solanacearum* lacking important virulence factors.
3. Virulence determination of mutants through functional assays or plant assays.
4. Validation of effector protein secretion through Type Three Secretion System.

CHAPTER II

Review of Literature



2.1. Bacterial Wilt

The crop production worldwide is reduced by 36% due to interference by insects, plant diseases and weeds. In this, the plant diseases alone counts for a share of 14% reduction in the crop yield (Agrios, 2005). Among the plant diseases, yield losses account for 10-20% due to soil-borne disease in comparison to seed-borne or air-borne diseases (USDA, 2003). Bacterial wilt is one such major disease which counts for huge losses in agriculturally important crops. Bacterial wilt is a general term used to describe the disease caused by many *spp.*, including the genus *Ralstonia, Clavibacter, Pantoea, Erwinia etc.* Bacterial wilt caused by *R. solanacearum* is reported widely and has a high destructive potential (Elphinstone, 2005; Hayward and Hartman, 1994).

The bacterial wilt is commonly associated with crops such as potato, tobacco, brinjal, pepper, tomato, chilli and peanut (Buddenhagen and Kelman, 1964; Sagar *et al.,* 2014; Ramesh *et al.,* 2014a). In addition to this, it is also observed in *Heliconia,* marigold, sunflower, mulberry, banana etc (Alvarez *et al.,* 2010). Bacterial wilt in ginger is reported in India since 1941 and since then has been diagnosed in Nigeria, Australia, Malaysia, Indonesia, South Korea, China, Hawaii, Mauritius, Philippines and Japan (Wubshet, 2018). In Indonesia, Florida and Central America severe bacterial wilt was observed in the virgin soils, where the crops were planted for the first time. This indicates that the bacterial wilt pathogen is persistent in the soils of various geographical separated areas (Buddenhagen and Kelman, 1964). The list of crops infected with bacterial wilt is increasing day by day.

2.2. Crop loss

The loss caused due to bacterial wilt varies depending on the climate, geography, soil type, cropping pattern, host and the strain (Nion and Toyota, 2015). Severe losses in yield has been reported globally, some of which include 88% of tomato in Uganda, 95% of tobacco in South Carolina, 50-100% of potato in Kenya and 100% on pepper in Ethiopia (Assefa *et al.*, 2015; Elphinstone, 2005). Major outbreak of bacterial wilt in ginger with disease incidence of 80-100% was reported by Kassa *et al*. (2015) in Ethiopia and 50-90% in Beefwood by Sun *et al*. (2013). In India, severe disease incidence is seen in chilli, tomato, brinjal, ginger and potato across various states of the country (Ramesh *et al.*, 2014a). Bacterial wilt is reported in the range of 60-80% among important crops like chilli, marigold, brinjal, tomato and ginger (Bholanath *et al.*, 2014).

Bacterial wilt can lead to heavy economic losses to small and marginal farmers who depend on agriculture as a main source of livelihood. *R. solanacearum* is referred as a quarantine pest (EPPO/CABI, 1996) by both the U.S.A. and the E.U. In U.S.A. It is also cited as bioterrorism organisms (Animal and Plant Health Inspection Service, 2002).

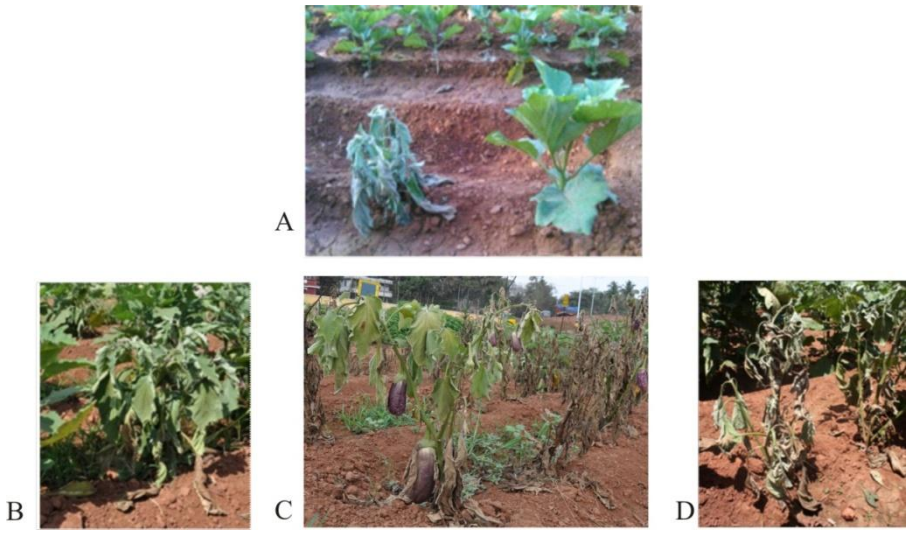


Fig. 2.1. Bacterial wilt caused by *R. solanacearum* in brinjal. (A) A wilted plant versus a healthy plant (B) Initial stage of wilt (C) Intermediate stage of wilt (d) Final stage of wilt

2.3. *Ralstonia solanacearum*

Ralstonia solanacearum (Yabuuchi *et al.,* 1995) is a soil borne vascular phytopathogen known globally to cause the devastating bacterial wilt in large number of agriculturally important crops. It belongs to the β-division of proteobacteria and infects a diverse range of more than 450 plant species and 54 botanical families (Wicker *et al.,* 2007) including the solanaceous plants, fruit crops, trees and weeds belonging to many monocot and dicot families.

In a survey of top 10 plant pathogenic bacteria published in the journal "Molecular Plant Pathology", *R. solanacearum* is listed on the second position and is referred as "probably the most destructive plant pathogenic bacterium worldwide (Mansfield *et al.,* 2012). In order to cause the lethal disease in the vascular system of the plant, *R. solanacearum* gains its entry through the roots and further penetrates the xylem as a result of extensive colonisation (Kelman, 1953).

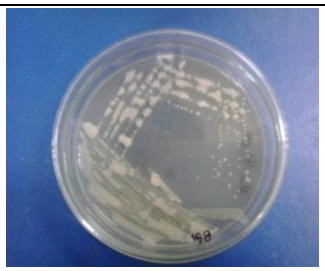


Fig. 2.2.*R. solanacearum*colonies on CPG/BG medium

2.4. Distribution and host range

The most important widespread hosts of *R. solanacearum* are brinjal (*Solanum melongena*), potato (*S. tuberosum*), groundnut (*Arachis hypogaea*), banana (*Musa paradisiaca*), *Heliconia* spp., tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon*

esculentum) with a majority of them belonging to the *Solanaceae* and *Musaceae* families (EPPO, 2004). *R. solanacearum* is predominant in the tropics, subtropical and warm temperate regions of the globe. However, even at lower temperatures some strains of *R. solanacearum* are able to infect tomato and potato plants and are spreading to temperate areas of the United States and Europe. Importation of the contaminated material is the main cause for dispersal of this bacterium (Champoiseau *et al.*, 2009; Elphinstone, 1996).

2.5. Taxonomy

Before assigning the name *R. solanacearum* to this pathogen, it was classified taxonomically in a series of different genus. This classification is as follows:

Smith (1896) originally identified this pathogen as *Bacillus solanacearum*. Later on, it was transferred to the genus *Pseudomonas* and was known as *Pseudomonas solanacearum* for quite a long time (Kelman, 1954). In the year 1992, Yabuuchi and co-workers shifted the bacterium into the genus *Burkholderia* based on analysis of phenotypic characters, cellular lipid and fatty acid composition, DNA-DNA homology values and 16s rRNA sequences. However, further analysis in the same line along with rRNA-DNA hybridisation experiment finally lead to the nomenclature of this organism into a new genus called "*Ralstonia*" and since then is now referred as *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995).

The group of *R. solanacearum* species are together called as "*Ralstonia solanacearum* species complex (RSSC)". The latest nomenclature proposed by Safni *et al*. (2014) using polyphasic taxonomic approach classifies the RSSC into three different genospecies. It subdivides the strains which belong to phylotype II as *R. solanacearum*, phylotype I and III as *R. pseudosolanacearum* and those of phylotype IV as *R. syzgii*.

The current classification of *R. solanacearum*is as follows:

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Burkholderiales

Family: Ralstoniaceae

Genus: *Ralstonia*

Species: *solanacearum*

(Source: www.ncbi.nlm.nih.gov)

2.6. Classification of *R. solanacearum*

*R. solanacearum*strains display high level of diversity between them. Due to this, the species is classified as "RSSC".

2.6.1. Biovar based classification

Based on the evaluation of the phenotypic traits, *R. solanacearum* is classified into six biovars (Fegan and Prior, 2005). Biovar classifies the strains based on the ability of the organism to metabolize or oxidize three disaccharides and to use three hexose alcohols (Fegan and Prior, 2005; Buddenhagen, 1986). The classification of *R. solanacearum* based on biovars is depicted in table 2.1.

Table 2.1. Biovar classification of *R. solanacearum* (EPPO, 2004; Hayward, 1991; Xue *et al.*, 2012).

Acid production from	Biovar					
	1	2	3	4	5	6
Cellobiose	-	+	+	-	+	+
Lactose	-	+	+	-	+	+
Maltose	-	+	+	-	+	+
Dulcitol	-	-	+	+	-	-
Mannitol	-	-	+	+	+	+
Sorbitol	-	-	+	+	-	+

2.6.2. Race based classification

Race classifies the strains based on the host range. *R. solanacearum*strains have been classified into five races. Race 1 has the largest host range. The race based classification is depicted in table 2.2.

Table 2.2. Differentiation of *R. solanacearum* strains into races based on the host range and geographical distribution. Adapted from (Buddenhagen, 1986, EPPO, 2004 and Alvarez *et al.*, 2010).

Race	Geographical distribution	Host range
1	Distributed throughout the lowlands of the tropics and subtropics, like Asia, Australia, America	Solanaceous crops like chili and sweet pepper, eggplant, potato, tobacco and tomato; Non-solanaceous crops like bean, groundnut and sunflower; Ornamental plants like *Anthurium* spp., *Dahlia* spp., H*eliconia* spp., *Hibiscus* spp., *Lesianthus* spp., *Lilium* spp., marigold, palms, *Pothos* spp., *Strelitzia* spp., *Verbena* spp. and *Zinnia* spp.; Trees like *Eucalyptus* and fruit trees as black sapote, custard apple, and Neem Others like abaca, cowpea, cucurbits, hyacinth beans, jute, moringa, mulberry, nutmeg, patchouli, *Perilla crispa*, sesame, strawberry, water spinach, wax apple and winged bean
2	Tropical areas of South America, Philippines, Indonesia and Viet Nam	Moko disease in bananas; Wild and ornamental *Heliconia* spp.
3	Widespread in all the five continents	Potato, geranium and tomato; Weeds like *Solanum dulcamara* and *S. nigrum*.
4	Asia	Ginger and the related plant species mioga and patumma;
5	China	Mulberry

2.6.3. Phylotype based classification

The recent classification based on molecular techniques groups the strains based on phylotypes or monophyletic clusters of strains using a single multiplex PCR. This classification is based on the similarity between the internal transcribed region (ITS) between the 16s-23S rRNA, *hrpB* and the *egl* (Fegan and Prior, 2005). The species is divided into four phylotypes which roughly corresponds to their geographical origin. Strains originating from Asia are classified as phylotype I, America as phylotype II, Africa as phylotype III and those from Indonesia, including *R. syzygii* and the Blood Disease Bacterium as phylotype IV (Fegan and Prior, 2005). The phylotypes are further divided into sequevars based on the sequences of *egl* and into clones by genetic fingerprinting techniques like rep-PCR and Pulsed Field Gel Electrophoresis (PFGE) (Ramesh *et al.,* 2014a).

This classification has been revised by Safni *et al*. (2014) into three geno-species based on phylogenetic analysis of 16s-23S rRNA ITS gene sequences, 16s-23S rRNA intergenic spacer (ITS) region sequences and partial *egl* gene sequences. It reclassifies the strains which belong to phylotype I and III as *R. pseudosolanacearum*, phylotype II as *R. solanacearum* and those of phylotype IV as *R. syzgii*. The classification of *R. solanacearum* into three species is also supported by Prior *et al* (2016), with the help of genomic, proteomic comparison and metabolic characterisation of the isolates.

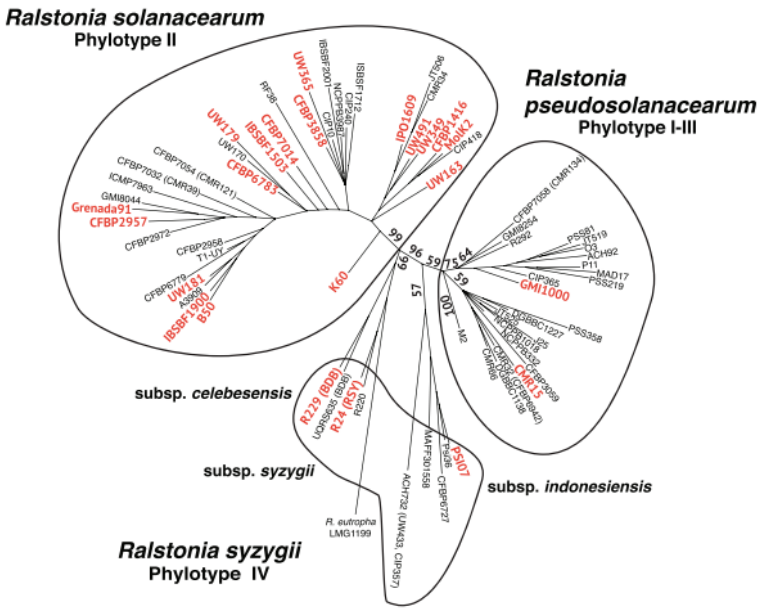


Fig. 2.3. Classification of *R. solanacearum* into three species

2.7. Phenotypic characteristics

*R. solanacearum*is a rod shaped Gram-negative bacterium with polar flagella (Smith, 1896; Kelman, 1953). The average size of a single cell varies from 0.5-0.7 by 1.5-2.5 µm (Denny and Hayward, 2001). It is aerobic in nature and produces poly-β-hydroxybutyrate granules as cell energetic reserve. The optimal growth temperature for most of the *R. solanacearum* strains is 28-32°C, however it has been reported to grow upto minimum of 8-10°C (Hayward, 1964; Schaad *et al.*, 2001; Alvarez *et al.*, 2010).

Morphologically *R. solanacearum* cells can form two different types of colonies: fluidal and non-fluidal. Fluidal colonies are produced due to the accumulation of extracellular polysacharride (EPS). On culturing the bacterium on Casamino acid peptone glucose (CPG) agar medium with tetrazolium chloride (TZC) well-formed irregular shaped white colonies

appear with characteristic pink whirls in the centre within 48h (Denny and Hayward, 2001). Under certain conditions the fluidal colonies may turn non fluidal and dark pink in color. This phenomenon is known as "Phenotypic Conversion" (PC) and leads to the loss in disease causing ability of the bacterium (Poussier *et al.*, 2003).

The most important diagnostic test for identification of BW is the bacterial streaming test (Elphinstone, 2005). The bacterial cells emerge out as a milky white ooze from the cut end of a stem and can be directly cultured as a pure culture on Triphenial Tetrazolum chloride (TZC) medium (Kelman, 1954). It permits the distinction between avirulent and virulent colonies on modified SMSA (Engelbrecht, 1994) which helps in suppression of the contaminants thereby permitting the detection of *R. solanacearum.*

2.8. Life cycle and infection process

2.8.1. Infection process in plants

Under favourable natural environmental conditions, *R. solanacearum* utilizes swimming motility (refer section 2.12.2.) to move towards the plants by sensing specific stimuli (Tans-Kersten *et al.*, 2001). The host root exudates which contains various organic acids and amino acids also attracts the bacterium (Yao and Allen, 2006), and thus *R. solanacearum* gains entry into the plant through the roots (Kelman, 1953). Once within the plant, it infects the cortex with the help of plant cell wall degrading enzymes (PCWDE). As the disease progresses, the susceptible plant displays symptoms of yellowing of the foliage and stunting externally (Kelman, 1953). Further, *R. solanacearum* moves into the xylem and initiates wilting which is controlled by a regulatory network that uses PhcA (refer section 2.11.1.) (Schell, 2000).

The infection process of *R. solanacearum* can be divided into two main stages of colonisation:

A) Root colonisation and cortex infection.

B) Colonisation in the xylem vessels.

A) Root colonisation and cortex infection: Presence of natural openings and/or physical wounds allows *R. solanacearum* to enter the roots (Vasse *et al.,* 1995). Generally, the axils of emerging or developed secondary roots which have weaker epidermal barrier and the root elongation zone which behaves as the major site for secretion of the root exudates acts as major sites for entry of *R. solanacearum* into the roots. *R. solanacearum* then moves further and invades the intercellular spaces in them and colonizes in the root cortex (Vasse *et al.,* 1995). The pectinolytic enzymes degrade the pectin polymers present in the middle lamella of the intercellular spaces and thus provide nutrients to the colonising *R. solanacearum* cells (Schell, 2000). The infection then proceeds to the inner levels of cortex.

B) Colonisation in the xylem vessels: *R. solanacearum* enters the vascular cylinder through the differentiating endodermis which is weaker in the root extremeties and axils of lateral roots (Vasse *et al.,* 1995). The cellulolytic enzymes possessed by *R. solanacearum* now play an important role and facilitates the cells accumulated around the stele to invade the xylem vessels (Schell, 2000). Observation in tomato plants have proved the presence of more than 10^{10} cells of *R. solanacearum* in the stem which travels to the upper parts in the plant (Vasse *et al.,* 1995). During this multiplication in the host, EPS (refer section 2.12.1.) plays a major role and blocks the transport of water thus leading to wilting of the plants. The degradation/discolouration of the internal vessels can be observed initially which progresses into necrosis causing the

plant to collapse and die thereby releasing *R. solanacearum* cells back into the soil (Kelman, 1953). In the soil, *R. solanacearum* leads a saprophytic life and waits for a new host.

2.8.2. Latent phase

The soils loaded with *R. solanacearum* cells acts as the source of inoculum for the infection in susceptible hosts. Bacterial cells released from the wilted plant debris maintain their full pathogenicity and can migrate down naturally to 75 cm of depth in the soil (Graham *et al*.,, 1979). In the warm and moist climate of the tropics, *R. solanacearum* is able to survive in the soil for long period of time (Elsas *et al.,* 2001). Many weeds also act as asymptomatic hosts and help in the multiplication of the bacteria within itself ultimately releasing them into the soil environment, example growth of *Solanum dulcamara* along the rivers (Persson, 1998). Quite often *R. solanacearum* cells persist in nearby water streams or ponds and enter into the nearby fields through irrigation. This represents the most dangerous and common source of inoculum (Hong *et al.,* 2008). It has been observed that, in irrigation waters, the bacterium is able to go through several replication cycles and maintain itself (Van Elsas *et al.,* 2001). The pathogenicity of *R. solanacearum* can also be maintained by storing it as sterile distilled water stocks in laboratory for up to four years (Buddenhagen and Kelman, 1964; Alvarez *et al.,* 2008). The long-term endurance of this pathogen in the soil can be attributed to the physiological survival mechanism such as the viable but not culturable form, possessed to overcome starvation (Alvarez *et al.,* 2008). This is a reversible state and the cells generally enter this state during the saprophytic phase of the disease cycle, when the temperature drops, or after completing the disease cycle in a plant (Grey and Steck, 2001).

2.9. Genome organisation

The first *R. solanacearum* strain to be sequenced for whole genome was GMI1000. It belongs to race 1, biovar 3 and phylotype I (Salanoubat *et al.,* 2002). The genome size of GMI1000 is 5.8 Mb and has a high percent of G+C content. The genome of *R. solanacearum* is distributed into two circular replicons: Chromosome and Megaplasmid. In GMI1000, the chromosome is 3.7 Mb and the megaplasmid is 2.1 Mb (Salanoubat *et al.,* 2002). Majority of the genes responsible for basic cellular functions are present on the chromosome while that of fitness of the bacterium are present on the megaplasmid (Salanoubat *et al.,* 2002).

2.9.1. Genome sequencing

Till date a total of 39 strains *of R. solanacearum* are sequenced globally from different phylotypes. Seven sequenced strains are isolated from India infecting the potato, *Zingiberaceae* plants and solanaceous vegetables (Ramesh *et al.,* 2014b; Patil *et al.,* 2017; Kumar *et al.,* 2017). Two strains of *R. solanacearum* infecting solanaceous vegetables are Rs-09-161 and Rs-10-244 (Ramesh *et al.,* 2014b). These strains belong to race 1, biovar 3, phylotype I and based on *egl* gene sequence analysis the isolates belong to two different representative subgroups (Ramesh *et al.,* 2014a; b). The variability seen with the various sequenced genome of *R. solanacearum* is probably due to transposable elements acquisition, loss and alteration of genetic material through transposable elements (Genin and Boucher, 2004).

2.10. Secretion systems in Gran negative bacteria

Gram negative bacteria have the presence of various secretion systems which help in the transport of virulence proteins outside the cell. These secretion systems are type I secretion

system (TISS), type II secretion system (T2SS), type three secretion system (T3SS), type IV secretion system (T4SS), type V secretion system (T5SS) and the type VI secretion system (T6SS). Each of this secretion system is involved in transporting a specific subset of proteins (Green and Mecsas, 2016).

2.10.1 The type I secretion system

The T1SS secretes substrates in a single step and is found in a large number of Gram-negative bacteria, including animal and plant pathogens (Thomas *et al.,* 2014). The T1SS resembles a large family of ATP-binding cassette (ABC) transporters and is involved in transporting substrates (Delepelaire, 2004). Virulence associated T1SS substrates are found in a variety of bacterial pathogens including *Vibrio* cholerae, *Serratia marcescens* and uropathogenic *E. coli* (Dolores *et al.,* 2015; Letoffe *et al.,* 1996; Hughes *et al.,* 1982).

2.10.2. The type II secretion system

The T2SS is involved in the transport of folded proteins from the periplasm into the extracellular environment. The substrates secreted through this system are delivered into the periplasm via the Sec or Tat secretion pathways and have the presence of Sec- or Tat-type cleavable signal sequence at their N termini (Korotkov *et al.,* 2012). Some of the T2SS substrates are involved in the virulence of the bacterial pathogen such as cholera toxin of *V. cholera* and exotoxin of *Pseudomonas aeroginosa* (Sandkvist *et al.,* 1997; Lu *et al.,* 1996). Some pathogens such as *Legionella pneumophila, Aeromonas hydrophila* secrete enzymes through the T2SS (Cianciotto *et al.,* 2013; Jiang *et al.,* 1992).

2.10.3. The type three secretion system

The T3SS is present across a large number of bacterial pathogens and symbionts (Green and Mecsas, 2016). The structure of this system resembles to that of injectisomes or needle and syringe apparatus (Green and Mecsas, 2016). They transport substrates across the bacterial membrane into the eukaryotic cytoplasm. These proteins are called as effectors and are present in large numbers in various pathogenic bacteria (Green and Mecsas, 2016). Many of the T3Es have homologs in various pathogens.

Xanthomonas spp., causes bacterial spots and blight in various crops and has the presence of approximately 40 T3E that promote its virulence. T3E XopJ is proved to be essential for pathogenicity of *X. campestris* (Noel *et al.,* 2003). XopJ effectors have acetyl transferase activity which is exerted on its target proteins. Homologs of XopJ effectors are also found in *Pseudomonas spp.*, *Yersinia pestis* and *R. solanacearum* wherein it functions in a similar manner (Ustun and Bornke, 2014; Peeters *et al.,* 2013). *P. syringae* use the T3Es AvrPtoB/HopAM1, HopQ1 and AvrRpt2 which interact with the abscisic acid (ABA) cytokinin and auxin signaling pathways respectively whereas the *X. campestris* strains use AvrXccC and AvrBs3 to induce ABA and auxin signaling, respectively (Marois *et al.*, 2002; Ho *et al.,* 2013; Cui *et al.,* 2013; Hann *et al.,* 2013).

2.10.4. Type IV secretion system

Bacterial conjugation is ancestrally related to the T4SS. The T4SS can secrete a variety of substrates including single proteins, protein-protein and DNA-protein complexes (Cascales and Christie, 2003). Like the T3SS, the T4SS can also transport its substrates into the eukaryotic cells. The plant pathogenic *Agrobacterium tumeficans* transports its oncogenic T-

DNA into the plant cells through the T4SS (Bundock *et al.*, 1995). Among the others like *L. pneumophila, Brucella suis*, and *Helicobacter pylori*, the T4SS is involved in the translocation of effector proteins (Green and Mecsas, 2016).

2.10.5. Type V secretion system

The TVSS secretes proteins only through the outer membrane. The proteins are secreted into the periplasm across the inner membrane by the Sec apparatus. The T5SS plays an important role in the pathogenesis of *Shigella flexneri* by the secretion of the IcsA protein, and *Ne*isseria *gonorrhoeae by* immunoglobulin A protease (Green and Mecsas, 2016).

2.10.6. Type VI secretion system

The T6SS is the most recently discovered secretion system in bacteria. This system is involved in translocating proteins in other bacteria as well as eukaryotic cells (Green and Mecsas, 2016). This system probably plays a role in interaction with the environment and in interbacterial competition (Green and Mecsas, 2016).

2.11. Virulence and pathogenicity regulatory network in *R. solanacearum*

2.11.1. *phcA* global regulator

PhcA is the virulence regulator in *R. solanacearum* which, responds to the cell density via quorum sensing called the phenotype conversion (*phc*) system. PhcA is a LysR-type transcriptional regulator and directly controls the expression of endoglucanases (*egl)* and the extracellular polysaccharide EPS (Schell, 2000; Brumbley *et al.*, 1993; Brumbley and Denny, 1990).

The *phcA* is controlled by the products of the *phcBSRQ* operon. It is activated by an autoinducer 3-hydroxypalmitic acid methyl ester (3OH-PAME) which is synthesized by *phcB* (Clough *et al.,* 1997b; Flavier *et al.,* 1997). In the absence of 3OH-PAME, *phcA* is repressed by the PhcS/PhcR two-component regulator system (Clough *et al.,* 1997b). PhcA regulator plays a central role in *R. solanacearum* under *in vitro* conditions, and allows the pathogen to change between two phenotypic states in response to nutrient availability and cell density (Denny, 2006; Schell, 2000). At low cell densities such as survival in soil or invasion of host plant, the concentration of 3-OH-PAME is low and therefore the *phcA* is repressed by the *phcS/phcR* two-component system. As a result, the genes coding for *egl* and EPS are repressed and those involved in twitching motility, swimming motility and attachment to the host tissue are expressed (Kang *et al.,* 2002; Liu *et al.,* 2001; Tans-Kersten *et al.,* 2001). During colonization in the susceptible host tissue, *R. solanacearum* reaches higher cell densities of 10^6 to 10^8 CFU.mL^{-1}, 3-OH PAME is synthesized which activates the *phcA*. The PhcA then directs the production of EPS and the secretion of *egl* (Schell, 2000). During this time, the expression of swimming motility and twitching motility is repressed (Allen *et al.,* 1997; Kang *et al.,* 2002; Schell, 2000; Tans-Kersten *et al.,* 2001).

Wild type strains of *R. solanacearum* with active *phcA* are highly virulent and produce large amount of EPS, whereas, whereas mutants of *phcA* are it avirulent and lacks EPS producing non fluidal colonies (Schell, 2000). *PhcA* mutants can arise spontaneously on agar plates incubated for long periods or in a still broth culture. Such mutants are unable to wilt host plants, but can colonize plants and causes stem necrosis, formation of adventitious roots and stunting of the plant (Poussier *et al.,* 2003). In addition to this, an increase in

polygalacturonase, siderophore and *hrp* secretion apparatus is also observed with inactivation of *PhcA* (Schell, 2000).

2.11.2. *vsrA/vsrD* two component system

The *vsrAD* two-component system is also known to control several traits in *R. solanacearum*. VsrAD promotes colonization in tomato stems and also activates the transcription of EPS (Schell, 2000; Genin and Denny, 2012). Mutation in *vsrAD* leads to reduced transcription of EPS genes. Inactivation of *vsrAD* also reduces the ability of *R. solanacearum*to rapidly colonize stems and multiply *in planta* and therefore loses the ability to cause any disease symptoms (Schell, 2000). VsrAD negatively controls swimming motility and twitching motility (Genin and Denny, 2012).

2.12. Virulence determinants of *R. solanacearum*

*R. solanacearum*has the presence of wide array of virulence factors which aid the bacterium in its virulence causing ability. These virulence factors include Extracellular polysaccharide (EPS), type two secretion system (T2SS) and cell wall degrading enzymes (CWDEs), swimming motility, chemotaxis, twitching motility and type three secretion system (T3SS). Besides these, the type six secretion system (T6SS) also plays a role in the virulence of *R. solanacearum* (Zhang *et al.*, 2012; 2014).

2.12.1. Extracellular polysaccharide

The macromolecule EPS produced in large quantities by the wild type strains is considered as the primary virulence factor of *R. solanacearum* and is known to impair the water transport

within its susceptible hosts (Schell, 2000). The first report of this massively produced fluid slime layer present around the colonies of *R. solanacearum* in vitro was reported by Kelman in 1954. It is secreted by seven genes *viz., epsA, epsB, epsC, epsD, epsF, epsP* and *epsR* (Asolkar and Ramesh, 2018a). This nitrogen rich acidic polymer EPS I, is heterogenous in nature and is constituted by trimeric repeat units of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose (Orgambide *et al.,* 1991). EPS deficient mutants are devoid of production of EPS in plants or *in vitro*. Phenotypically visualized as non-mucoidal colonies, they are non-pathogenic in nature (Orgambide *et al.,* 1991). Pathogenicity studies using EPS deficient mutants have demonstrated them to be poorly infective and incapable of invading xylem vessels (Araud-Razou *et al.,* 1998; Orgambide *et al.,* 1991).

2.12.2. Swimming motility

Polar flagella mediated swimming motility plays an important role in the virulence of *R. solanacearum* (Tans-kersten and Allen, 2001). Polar flagella is composed of flagellin subunit FliC which varies from 1-4 and helps propel itself towards host rhizospere and invade the root tissue during early stage of bacterial wilt (Tans-Kersten and Allen, 2001). Swimming motility is associated with cell density in plants as well as in culture and is exhibited at a concentration of 10^8 CFU.mL^{-1} (Clough, *et al.,* 1997a; Tans-Kersten and Allen, 2001). Wild type virulence is displayed by the non-motile *fliC* mutant when inoculated directly onto the vascular system in tomato but is highly reduced in disease causing ability through soil soak inoculation (Tans-kersten and Allen, 2001). Importance of flagellum-mediated motility was proved with the *motN* mutant which is a negative regulator of motility. The *mot N* mutant was found to be

hyper motile and hyper flagellated but significantly reduced in virulence on tomato in soil soaked inoculation (Meng *et al.,* 2011).

2.12.3. Chemotaxis

Specific chemicals present in the environmental conditions are sensed by many bacteria by a process of taxis which helps in movement of the bacteria towards the host by attraction or away from non-host by repulsion (Adler, 1966; Blair, 1995). *R.* solanacearum possesses this feature of chemotaxis. In *E. coli*, chemotaxis has been well studied along with its mechanism of response regulation and signal transduction (Stock and Surette, 1996). *R. solanacearum* gets attracted towards different organic acids and amino acids (Yao and Allen, 2006). Yao and Allen found that *R. solanacearum* gets attracted more towards the root exudates of host plants like tomato than that of non-host plant like rice. Chemotactic ability of *R. solanacearum* plays an important role in host invasion, as the mutants have reduced virulence under natural infecting conditions but display full virulence when introduced directly into the vascular tissue (Yao and Allen, 2006).

2.12.4. Twitching motility

R. solanacearum exhibits twitching motility on solid surfaces. Twitching motility is associated with the type IV pili and plays an important role in autoaggregation and biofilm formation. Twitching motility helps *R. solanacearum* to adhere to the host cells and also play an important role in natural transformation (Kang *et al.*, 2002). The genes *pilA* and *pilP* are associated with twitching motility and contribute to type 4 fimbrial pilin signal peptide protein and fimbrial type-4 assembly lipoprotein respectively. The type IV pili is made up of monomers of *pilA* protein (Kang *et al.,* 2002). The type IV pili contribute towards the

virulence of *R. solanacearum* and the mutants of twitching motility are attenuated in its virulence on susceptible tomato plants (Liu *et al.,* 2001). The *pilQ* and *pilT* play an important role in the formation of *Tfp,* mutants of these caused slower and less severe wilting on tomato plants (Liu *et al.,* 2001).

2.12.5. Type two secretion system and cell wall degrading enzymes

The CWDEs encoded by the T2SS plays an important role in the pathogenesis of *R. solanacearum* by hydrolysing complex carbohydrates present as plant cell wall components (Liu *et. al*., 2005). In addition to the CWDEs, the ~24 other extracellular proteins secreted by the T2SS also play a vital role in the virulence of the bacterium (Genin and Denny, 2012). The CWDEs consists of two cellulytic (cellobiohydrolase-*cbhA* and endoglucanase-e*gl*) and four pectic (endo-polygalacturonase-*pehA*, di-exo-polygalacturonase-p*ehB*, exo-polygalacturonase-*pehC* and pectin methyl esterase-*pme*) enzymes (Liu *et al.,* 2005; Pouymeiro and Genin, 2009).

By degrading the cell wall components, bacterium obtains nutrients and energy in addition to facilitating its entry and spread in the host plant (Huang and Allen, 2000). The cellulytic enzyme *cbhA* and *egl* contribute additively and make largest contributions to virulence among CWDEs on tomato plants (Liu *et al.,* 2005). The *in planta* transcriptomic analysis revealed *cbhA* to be among highly expressed genes of *R. solanacearum* during tomato infection (Jacobs *et al.,* 2012).

Pectinase enzyme coded by the bacterium may play an important role in colonisation by degrading the pectin gels present surrounding the lateral roots and xylem vessels. Presence of *pme* allows the bacterium to grow on methylated pectin by removing methyl groups from

pectin. *pme* therefore is not directly involved in the virulence on tomato but facilitates the availability of pectin for breakdown by *pehA, pehB* and *pehC* (Liu *et al.,* 2005; Tans-Kersten and Allen, 1998). The *pehA/pehB* double mutant has an additive defect on virulence and hence decreases the virulence of pathogen on tomato plants (Gonzalez and Allen, 2003). However, *pehA/pehB/pehC* triple mutant is more virulent than the double mutant. This is because, the plant defence triggering oligogalacturonides produced by *pehA/pehB* are degraded by the *pehC* gene (Gonzalez and Allen, 2003; Liu *et al.,* 2005).

2.12.6 Type six secretion system

The T6SS is the most recently discovered secretion system in bacteria. The T6 secretion gene cluster is widely present in the genome of many gram negative bacteria, especially proteobacteria (Boyer *et al.,* 2009; Cascales and Cambillau, 2012). The T6SS plays several roles in bacteria. Initially it was thought to be only involved in pathogenesis, but recent studies have exhibited its importance in bacterial interactions and competition. The T6SS interacts with both prokaryotic and eukaryotic cells (Silverman *et al.,* 2012). The T6SS secretes anti-bacterial proteins into the target cells and thus kills the neighbouring, non-immune bacterial cells by cell-to-cell contact (Cascales and Cambillau, 2012).

Like the T3SS, the T6SS is also involved in translocation of substrates into the recipient cells through contact dependent manner (Silverman *et al.,* 2012; Cornelis, 2006). In case of eukaryotes, it secretes toxin molecules which interfere with the eukaryotic cytoskeleton and thus plays a role in pathogenesis (Cascales and Cambillau, 2012). The T6SS injects toxins into the cell membrane or cytoplasm of the prey through a contractile nanomachinery. This contractile nanomachinery is assembled in orderly manner to form a platform for baseplate formation which acts as a base for contractile tail elongation (Gallique *et al.,* 2017). The T6SS

gene cluster is also present in *R. solanacearum* and is necessary for virulence of *R. solanacearum* on tomato (Zhang *et al.,* 2012; 2014).

2.12.6.1. Structure of the T6SS

The T6SS is formed by assembly of two distinct substructures: an inverted bacteriophage like injection apparatus and a membrane complex. The membrane complex interacts with the inverted bacteriophage like structure and anchors it into the cell envelope. The secretion apparatus is made up of 13 core components which are supplemented with additional proteins (Cascales and Cambillau, 2012). The core components are named from *tssA* to *tssM*, and many of them share homology with bacteriophage proteins (Badr *et al.,* 2016). The core components assemble together to form a structure that shares similarity with inverted bacteriophage like structure anchored to the bacterial cell envelope (Records, 2011; Kanamaru, 2009).

2.12.6.2. The Membrane complex:

The membrane complex forms a base onto which the inverted bacteriophage apparatus assembles in the cell envelope. Along with formation of a platform for assembly of the baseplate complex, the membrane complex also prevents the damage to the cell membrane during effector injection (Aschtgen *et al.,* 2008).

It is 1.7 MDa in size and made up of the heterotrimeric protein complex of three core component proteins: TssM, TssL and TssJ, along with the accessory protein TagL (Gallique *et al.,* 2017). The TssJ is a lipoprotein which localises in the outer membrane, TssM and TssL localise at the inner membrane. TssM links the inner membrane with the outer membrane by interacting with TssL and TssJ (Ma *et al.,* 2011). TssM interacts with TssJ through its

periplasmic domain and with TssL through its cytoplasmic domain (Felisberto-Rodrigues *et al.,* 2011; Logger *et al.,* 2016).

The peptidoglycan layer present in the cell membrane is degraded by lytic transglycosylase (LTG) which help in insertion of the membrane complex in the cell envelope. LTG is recruited by the periplasmic domain of TssM (Weber *et al.,* 2016). TagL has the presence of a functional peptidoglycan-binding (PGB) domain and is inserted into the inner membrane through three transmembrane segments. TagL interacts directly with the TssL. The PGB domain of TagL is required for the activity of the T6SS (Silverman *et al.,* 2012).

2.12.6.3. The Bacteriophage like Injection Apparatus:

The bacteriophage injection apparatus consists of the baseplate complex and the contractile tail complex. The baseplate complex assembles and positions itself over the membrane complex and serves as a platform for contractile tail and tail sheath assembly (Gallique *et al.,* 2017).

2.12.6.4. The baseplate complex:

The baseplate complex is recruited by the membrane complex and is composed of TssA, TssE, TssF, TssG, TssK, and VgrG (Valine-glycine repeat protein G) proteins. VgrG is also known as the TssI protein (Brunet *et al.,* 2015; Zoued *et al.,* 2013). A connection between the membrane complex and the T6SS contractile tail is formed by the baseplate complex. It is essential for the correct assembly of the inner tube of the contractile sheath (Brunet *et al.,* 2015).

Basler *et al.* (2012) showed the presence of putative baseplate in *Vibrio Cholerae* in the form of bell-shaped density through electron cryo-tomographs. TssA first binds to the membrane

complex and forms a dodecamer complex and initiates the positioning of the baseplate complex by recruitment of TssE, TssK, and VgrG via TssA (Zoued *et al.,* 2016). VgrG is a trimeric protein which helps in assembly of the tail sheath and stacking of Hcp hexamers. TssE helps in polymerisation of TssBC sheath (Zoued *et al.,* 2014). VgrG shares structural similarity with gp27/gp5 which forms the tail spike in T4 and TssE is homologous to bacteriophage wedge gp25 (Cascales and Cambillau, 2012). At the end of the *VgrG* protein, PAAR (Pro-Ala-Ala-Arg) repeat containing proteins bind to form a sharp tip of *HCp-VgrG* (Gallique *et al.,* 2017). The N-terminal region of VgrG3 (VgrG3N) forms an important component of T6SS apparatus and a C-terminal domain (VgrG3C) functions as a peptidoglycan-targeting glycoside hydrolase and is released as an effector in *Vibrio cholera* (Brooks *et al.,* 2013).

2.12.6.5. Contractile tail complex:

The T6SS contractile tail complex consists of an inner tail tube and the outer tail sheath (Zoued *et al.,* 2014). The inner tail tube is made up of Hcp proteins and is formed under the control of baseplate components. Hcp proteins are arranged in the form of hexameric rings stacked head-to-tail with an outer diameter of 80-90A°. The tube is hollow on the inner side with a diameter of 40A°. This gap can accommodate an unfolded/partly folded or small folded protein (Cascales and Cambillau, 2012). The tertiary structure of Hcp proteins shares similarity with bacteriophage λ tail tube protein gpV (Zoued *et al.,* 2014).

The T6SS tail sheath is made up of TssB and TssC proteins and shares structural similarity with bacteriophage sheath. It has a diameter of 100-110A° and wraps the inner tail tube (Zoued *et al.,* 2014). Immunogold labelling of TssB by Kapitein *et al.* (2013) has confirmed its role in T6SS sheath formation. The *tssB* mutant of *R. solanacearum* has significantly

attenuated virulence on tomato, decreased motility and defect in biofilm formation (Zhang *et al.,* 2014).

2.13. Pathogenicity determinant of *R. solanacearum*

2.13.1. Type three secretion system (T3SS)

The T3SS is an essential pathogenicity determinant and is encoded by the hypersensitive response and pathogenicity (*hrp*) regulon in *R. solanacearum* (Boucher *et al.,* 1987). The *hrp* regulon induces a hypersensitive response (HR) in non-host or resistant plants and pathogenicity in the susceptible plants (Hueck, 1998) and a defect in the T3SS results in the loss of the ability to induce both; a hypersensitive response and pathogenicity in plants (Alfano and Collmer, 2004). A hypersensitive response in characterised by localized necrosis which prevents the spread of bacterium to the other parts of the plant (Alvarez *et al.,* 2010).

The T3SS is activated *in planta* in a contact dependant manner by an unidentified non-diffusible cell wall component by the outer membrane receptor PrhA (Aldon *et al.,* 2000). This activates a cascade of transcriptional regulators whose final destination is the *HrpB* regulator (Brito *et al.,* 2002). PrhA activates the transmembrane sensor protein PrhR, which further activates the ECF sigma factor PrhI, which is the able to induce *PrhJ* expression (Brito *et al*. 1999; Marenda *et al.,* 1998). The virulence of *Prh* (plant regulatory hrp) mutants is mildly attenuated in tomato plants (Brito *et al.,* 1999; Genin *et al.,* 2005). PrhJ activates an OmpR family regulator *hrpG* which finally activates *hrpB* (Brito *et al.,* 2002). In invitro conditions, the T3SS is activated by *hrpG* and a paralog *prhG* (Plener *et al.,* 2010). In addition to this, *hrpG* also regulates eps production, biosynthesis of plant hormone analogs

and amino acids and resistance to reactive oxygen species (ROS) (Valls *et al.,* 2006). The *hrpB* is an AraC type transcriptional regulator and regulates the expression of *hrp* genes and known T3SS substrates. Mutant of *hrpB* and *hrpG* are avirulent and display limited multiplication in the intercellular spaces of the root cortex and vascular system of tomato and brinjal (Asolkar and Ramesh 2018b; Vasse *et al.,* 2000). Analysis of the hrp regulated promoters has led to the identification and characterization of a 25-nucleotide consensus sequence, called the hrp_{II} box (TTCGn16TTCG) in the hrp regulated genes of *R. solanacearum*. The position of this regulatory element is found to be conserved at −70 to −47 bp from the transcriptional start (Cunnac *et al.,* 2004a). Similar regulatory element was previously identified in hrp promoters of *Xanthomona*s and referred as PIP box (plant inducible promoter box) (Fenselau and Bonas, 1995). The expression of the T3SS was considered very important only during early stages of infection in *R. solanacearum* to suppress host the host immunity. But recent *in planta* transcriptome studies have proved its importance throughout the disease cycle (Jacobs *et al.,* 2012; Monteiro *et al.,* 2012b).

2.13.2. Type three effectors

T3SS works in a highly efficient manner to perform its primary function of translocation of various secretory proteins termed as “effectors” into the plant cytosol on contact of the bacterium with the plant tissue. Once into the host, it manipulates host cellular processes and subverts them for its own benefit. The effectors act as toxins and target the host immune system. In order to translocate the effectors into the plant cytosol the T3SS has to cross multiple barriers including the two bacterial membranes separated by a peptidoglycan layer and the thick cell wall and the plasma membrane of the plant cell (Bretz and Hutcheson, 2004). This translocation is brought about by Hrp dependant filamentous structure called as

hrp pili (Van Gijsegem *et al*., 2000; 2002). In situ immunogold labelling experiments suggest that the hrp pili acts as a needle to provide a protein transport channel for transport of effector proteins into the host cytosol. All the hrp mutants that lack the *Hrp* pilus protein (*hrpY*) cannot secrete Hrp substrate proteins like hairpins and effectors (Van Gijsegem *et al.,* 2002).

The characterization of *hrpII* box regulatory element made it easy for identifying the repertoire of candidate Type III effectors from the sequenced strains of *R. solanacearum* all over the world with the use of bioinfomatics approaches. A total of 110 T3Es are identified and annotated based on the presence of *hrpII* box. The annotated T3E genes are assigned the generic name Rip (Ralstonia injected protein) genes (Peeters *et al.,* 2013). Individual *R. solanacearum* strains have an average of 70-75 T3E, which is much larger than many other bacterial plant pathogens like *P. syringe* and *Xanthomonas sp.* wherein it is in the range of 30-40 (Hajri *et al.,* 2011; Zumaquero *et al.,* 2010). Hence, it is presumed that an ancestor of *R. solanacearum* probably possessed a large number of effectors because the majority of the strains possess a high number of effectors.

The only exception to this is BDB and has less number of effectors (Genin and Denny, 2012). Comparison of the T3E repertoire of ten strains of *R. solanacearum* has revealed the presence of 32 core effectors in them (Peeters *et al.,* 2013). Many of the T3Es are present in the form of gene families. The families include Rip A (AWR) family, Rip G (GALA) family, Rip H (HLK) family, Rip S (SKWP) family and Rip P (Pop) family. The family contains three to eight effector genes (Mukaihara *et al.,* 2010; Poueymiro and Genin, 2009; Remigi *et al.,* 2011; Sole *et al.,* 2012; Peeters *et al.,* 2013). These effectors have various internal repeats within them which are probably involved in interacting with the plant to mediate protein-protein or DNA binding activity (Poueymiro and Genin, 2009). A large number of T3E genes

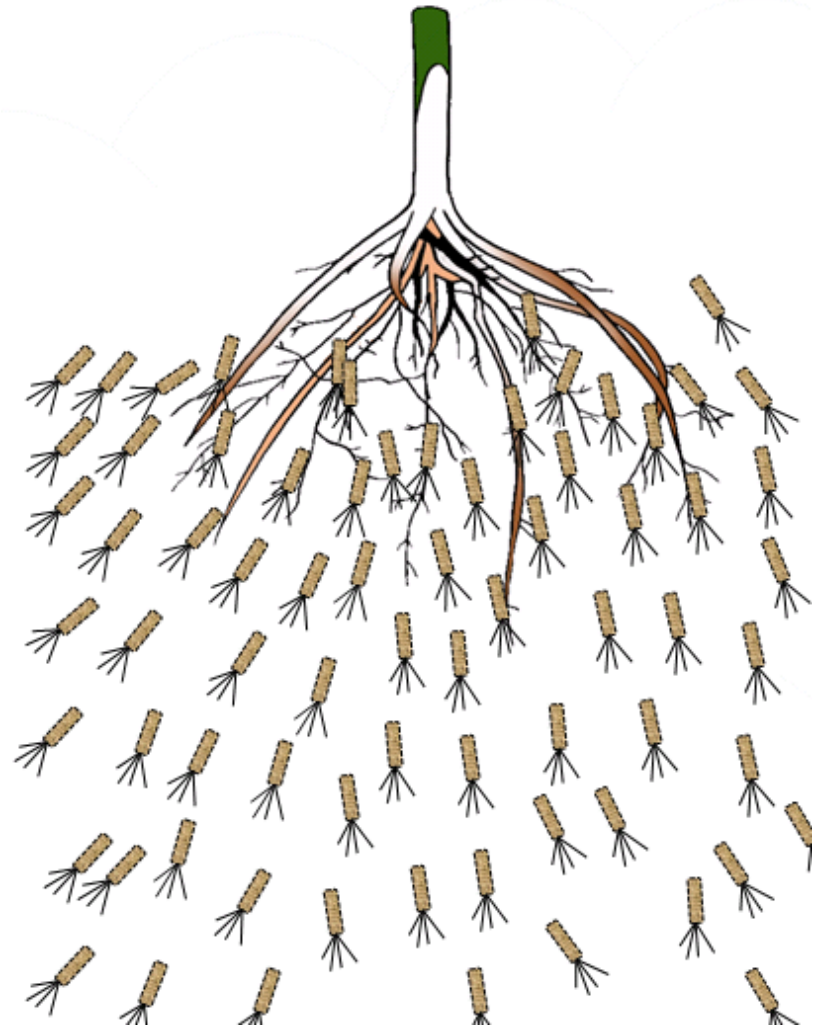
are validated to be translocated into the plant through the T3SS with the help of reporter based systems (Cunnac *et al.,* 2004b; Mukaihara *et al.,* 2009; 2010; Monteiro *et al.,* 2012a).

2.14. Management of bacterial wilt

Various management strategies have been investigated in bacterial wilt. These include physical, chemical, biological and cultural methods. Many of these strategies have shown limited success in the management of bacterial wilt (Ramesh *et al.,* 2016). These strategies include soil amendments (Islam and Toyota, 2004), soil solarization (Kumar and Sood, 2001), use of bio-fumigants (Pradhanang *et al.,* 2003), resistant plant (Dalal *et al.,* 1999), transgenic resistant plant (Jia *et al.,* 1999), SAR inducers (Anith *et al.,* 2004), plant growth promoting rhizobacteria (Singh *et al.,* 2012) and biological control (Ramesh and Phadke, 2012; Achari and Ramesh, 2014). Recently, Ramesh *et al.* (2016), reported grafting as a promising management strategy in controlling bacterial wilt with the help of *Solanum torvum* root stock. Field evaluation of these grafts indicated complete survival and optimal yield whereas that of seedlings displayed 60-80% wilt. Grafting can be considered as a future management strategy against bacterial wilt.

CHAPTER III

Prediction of probable virulence factors, effector proteins in *Ralstonia solanacearum* through genomic analysis.



3.1 Introduction

Ralstonia solanacearum causes bacterial wilt in many crop plants and is responsible for huge losses in agriculturally important crops (Genin and Denny, 2012). *R. solanacearum* finds its way into the plant through wounds in the roots and initiates wilting by impairing transport of water in the xylem which ultimately leads to the death of the infected plant (Genin and Denny, 2012).

Exopolysacharide (EPS) produced by the bacterium is the primary virulence factor and impairs water transport within its susceptible host (Schell, 2000). In addition to EPS, the Type Two Secretory System (T2SS), chemotaxis, swimming and twitching motility also contribute towards the virulence of the bacterium (Saile *et al.,* 1997). The T2SS secretes various plant cell wall degrading enzymes (PCWDE) that include cellulytic and pectinolytic enzymes which promote the colonization of the bacterium in the plant tissue. Chemotaxis and twitching motility also act as important virulence factors and aid in locating and attaching of the bacterium to the host roots (Alvarez *et al.,* 2010; Genin and Denny, 2012).

The T3SS is an essential pathogenicity determinant and is encoded by the *hrp* (hypersensitive response and pathogenicity) regulon in *R. solanacearum* (Boucher *et al.,* 1987). The *hrp* regulon is so named because it induces a hypersensitive response (HR) in non-host or resistant plants and pathogenicity in the susceptible plants (Hueck *et al.,* 1998). A defect in the T3SS results in the loss of the ability to induce both; a hypersensitive response and pathogenicity in plants (Alfano and Collmer, 2004). The T3SS enables a bacterium to translocate pathogenicity proteins called as “Type Three effectors (T3E)” into the cytosol of eukaryotic host cells. The effectors act as toxins and target the host immune system. The expression of *R.*

solanacearum T3SS is induced in the presence of poor nutritional conditions which mimics that of intracellular spaces in plants (Genin *et al.,* 1992).

The draft genome sequence of two *R. solanacearum* isolates Rs-09-161 and Rs-10-244 was published recently (Ramesh *et al.*, 2014b). These isolates belong to India and are maintained at the Plant pathology laboratory of ICAR- Central Coastal Agricultural Research Institute (ICAR-CCARI), Goa. Since the virulence factors and T3E contribute to the virulence of *R. solanacearum*, this study was taken up with the objective of "**Prediction of probable virulence factors, effector proteins in *Ralstonia solanacearum* through genomic analysis".**

3.2 Materials and Methods

3.2.1 *R. solanacearum isolates*

Ralstonia solanacearum isolates Rs-09-161 and Rs-10-244 were selected to analyze the virulence factors and T3Es. These are the whole genome sequenced isolates and belong to Phylotype I from India (Ramesh *et al.,* 2014b) and are being maintained in the culture collection of Plant Pathology lab, ICAR-CCARI, Goa. The isolates were tested for the production of EPS, twitching motility, cellulase and pectinase using standard procedures (Appendix I). Annotation of various virulence genes of *R. solanacearum* Rs-09-161 and Rs-10-244, was carried out using Eugene-P (Foissac *et al.,* 2008), with GMI1000 (Phylotype-I) as standard reference isolate. The general features of all *R. solanacearum* isolates used in this study are given in the Table 3.1.

Table 3.1 *R. solanacearum* isolates used in this study

Isolate	Phylo type	Geographical origin	Isolated from	Genome Size(Mb)	Total no. of T3Es	Hypothetical effectors	Accession	Remarks/Purpose in this study
Rs-09-161	I	India	Brinjal	5.65	72	3	PRJNA217471	Test isolate
Rs-10-244		India	Chilli	5.66	77	1	PRJNA236788	Test isolate
GMI1000		French Guyana	Tomato	5.811	71	1	PRJNA13	Virulence factors , T3E
RS1000		Japan	Tomato	NA	65	0		T3E
Y45		China	Tobacco	5.712	50	3	PRJNA182081	T3E
FQY_4		China	Bacterial Wilt Nursery	5.805	52	2	PRJNA182081	T3E
CFBP2957	IIA	French West Indies	Tomato	5.683	72	5	PRJEA50685	Virulence factors , T3E
IPO1609	IIB	Netherlands	Potato	5.313	60	5	PRJNA32087	T3E
UW551		Kenya	Geranium	5.895	58	3	PRJNA15601	T3E
Molk2		Philippines	Banana	5.961	76	8	PRJNA32085	T3E
Po82		Mexico	Potato	5.43	75	4	PRJNA66837	Virulence factors , T3E
CMR15	III	Cameroon	Tomato	5.593	68	5	PRJEA50681	Virulence factors , T3E
Psi07		Indonesia	Tomato	5.606	72	7	PRJEA50683	Virulence factors , T3E
BDB R229	IV	Indonesia	Banana	5.159	57	4	PRJNA53877	T3E
R. syzygii R24		Indonesia	Clove	5.424	48	2	PRJNA53879	T3E

Information was compiled based on the published literature and NCBI database

3.2.2 *Analysis of virulence factors*

Various virulence genes involved in the colonization and wilting of the host were identified in the isolates Rs-09-161 and Rs-10-244 based on the annotation data. These include the genes coding for exopolysaccharide-*EPS* (*epsA, epsB, epsC, epsD, epsE, epsF, epsP and epsR*), plant cell wall degrading enzymes-PCWDE (*pehA, pehB, pehC, pme, egl and cbhA*), chemotaxis (*cheA* and *cheW*), Swimming motility (*fliC* and *flgM*) and twitching motility (*pilA* and *pilP*). The coding sequences of these virulence genes were compared with representative isolates of *R. solanacearum* from different phylotypes *viz*. GMI1000 (phylotype-I), CFBP2957 (phylotype IIA), Po82 (phylotype IIB), CMR15 (phylotype PIII) and Psi07 (phylotype IV). The nucleotide sequences for virulence factors of Rs-09-161 and Rs-10-244 were retrieved from annotated files and of the reference isolates were extracted from NCBI database (www.ncbi.nlm.nih.gov). Virulence sequences were submitted to Genbank and accession numbers were obtained (Table 3.2). The sequences were aligned pairwise using Clustal W (Thompsons, 1994) and the evolutionary similarity matrix was constructed using MEGA version 6 software with p-distance method and bootstrap value of 1000 (Tamura *et al.*, 2013).

Table 3.2. Identification of various virulence factors in *R. solanacearum*: Rs-09-161, Rs-10-244, GMI1000

Gene Name	Rs-09-161 Locus tag*	Accesion No.	Rs-10-244 Locus tag*	Accesion No.	GMI1000 Locus tag	Location	Gene description
Exopolysacharide							
epsA	RALSO161_m00209880	KY661391	RALSO244_m00210550	KY661392	Rsp1020	Megaplasmid	EPS I polysaccharide export outer membrane transmembrane protein
epsB	RALSO161_m00209860	KY661393	RALSO244_m00210530	KY661394	Rsp1018	Megaplasmid	EPS I polysaccharide export transmembrane protein
epsC	RALSO161_m00209850	KY661395	RALSO244_m00210520	KY661396	Rsp1017	Megaplasmid	udp-n-acetylglucosamine 2-epimerase protein
epsD	-	-	-	-	-	-	Absent in Phylotype I isolates
epsF	RALSO161_m00209820	KY661399	RALSO244_m00210490	KY661400	Rsp1014	Megaplasmid	EPS I polysaccharide export inner membrane transmembrane protein
epsE	RALSO161_m00209830	KY661397	RALSO244_m00210500	KY661398	Rsp1015	Megaplasmid	EPS I polysaccharide export inner membrane transmembrane protein
epsP	RALSO161_m00209870	KY661401	RALSO244_m00210540	KY661402	Rsp1019	Megaplasmid	low molecular weight protein-tyrosine- phosphatase
epsR	RALSO161_m00203460	KY661403	RALSO244_m00203750	KY661404	Rsp0338	Megaplasmid	negative regulator of exopolysaccharide production transcription regulator protein

Gene Name	Rs-09-161 Locus tag*	Accesion No.	Rs-10-244 Locus tag*	Accesion No.	GMI1000 Locus tag	Location	Gene description
Plant Cell Wall degrading enzymes(PCWDE)							
pehA	RALSO161_m00208600	KY661405	RALSO244_m00209360	KY661406	RSp0880	Megaplasmid	polygalacturonase precursor (pectinase) signal peptide protein
pehB	RALSO161_m00118040	KY661407	RALSO244_m00117610	KY661408	RSc1756	Chromosome	exo-poly-galacturonase signal peptide protein
pehC	RALSO161_m00208120	KY661409	RALSO244_m00208880	KY661410	RSp0833	Megaplasmid	polygalacturonase transmembrane protein
pme	RALSO161_m00201470	KY661411	RALSO244_m00201620	KY661412	RSp0138	Megaplasmid	putative pectinesterase
egl	RALSO161_m00201640	KY661413	RALSO244_m00201830	KY661414	RSp0162	Megaplasmid	endoglucanase precursor (endo-1,4-BETA- glucanase) protein
cbhA	RALSO161_m00205550	KY661415	RALSO244_m00206300	KY661416	Rsp0583	Megaplasmid	exoglucanase A
Chemotaxis							
cheA	RALSO161_m00213570	KY661427	RALSO244_m00214120	KY661428	Rsp1408	Megaplasmid	chemotaxis sensor histidine kinase transcription regulator protein
cheW	RALSO161_m00213560	KY661417	RALSO244_m00214110	KY661418	Rsp1407	Megaplasmid	chemotaxis protein CheW

Gene Name	Rs-09-161 Locus tag*	Accesion No.	Rs-10-244 Locus tag*	Accesion No.	GMI1000 Locus tag	Location	Gene description
Swimming motility							
fliC	RALSO161_m00203910	KY661423	RALSO244_m00204190	KY661424	Rsp0382	Megaplasmid	flagellin protein
flgM	RALSO161_m00203480	KY661425	RALSO244_m00203770	KY661426	Rsp0340	Megaplasmid	negative regulator of flagellin synthesis (anti- sigma-28 factor) protein
Twitching motility							
pilA	RALSO161_m00105750	KY661419	RALSO244_m00105800	KY661420	Rsc0558	Chromosome	type 4 fimbrial pilin signal peptide protein
pilP	RALSO161_m00130530	KY661421	RALSO244_m00129280	KY661422	Rsc2972	Chromosome	fimbrial type-4 assembly lipoprotein

*Locus tag of Rs-09-161 and Rs-10-244 is the tag provided during assembly and annotation of the genome data

3.2.3 Identification of T3E

The preliminary identification of T3E genes was carried out by screening for the presence of *hrpII* box element (TTCGn16TTCG) in the region 500 bp upstream of the start codon using PatScan wherein only one mismatch was allowed. The presence of T3SS dependant export pattern in the T3E genes was detected by analysis of the 50 amino acid N terminal domain. The T3E was considered positive for N-terminal domain if it fulfilled atleast two out of the three criterias mentioned below: (a) Serine + Proline content should be greater than 30% (b) Leucine content should be lesser than 10% (c) Acidic residues should be absent within the first twelve amino acids.

Prediction of the start codon of the gene was carried out by the multiple sequence alignment of the region located downstream of the *hrpII* box element. The more distal 5'initiator codon conserved among different isolate sequences was considered as the start codon. The predicted T3E genes were also analysed for frame shift mutations and pseudogenes. T3E genes which had open reading frames disrupted by the insertion of IS element, altered structure (<50%) of the gene or evidence that the T3E gene product is not translocated by the T3SS were considered as Pseudogenes (Peeters *et al.*, 2013). The identification of candidate T3Es in the genomes of Rs-09-161 and Rs-10-244 was carried out using "Scan Your Genome" (Peeters *et al.*, 2013).

3.2.4 Analysis of the T3Es

The phylogenetic analysis based on the gene families of T3Es of *R. solanacearum* was studied. The gene families analysed include RipA (*AWR* family), RipG (*GALA* family), RipH

(*HLK* family) and RipS (*SKWP* family). The coding sequences of effectors belonging to each gene family were arranged in concatenated manner and compared with other isolates. Reference isolates used to study the phylogenetic relation of T3E are indicated in Table 1. The sequences of the T3Es of *R. solanacearum* isolates are available at https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev3/. Phylogenetic analysis was performed in MEGA version 6.0 (Tamura *et al.,* 2013) by using Neighbor-Joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap re-samplings.

3.3 Results and Discussion

3.3.1 *R. solanacearum* isolates

In India *R. solanacearum* has been isolated from various agriculturally important crops like brinjal, chilli, ginger, tomato, potato, capsicum etc. (Kumar *et al.,* 2004; Chandrashekara *et al.,* 2012; Ramesh and Phadke, 2012). Even though the bacterial wilt is a severe issue and affects various crops in India, there are only two isolates (Rs-09-161 and Rs-10-244) infecting solanaceous vegetables sequenced from India. Various virulence factors *viz.,* production of EPS, endoglucanase, pectinase and twitching motility of Rs-09-161 is depicted in Fig. 3.1. With the availability of genomic data through whole genome sequencing, it has become interesting to study *R. solanacearum* at the genomic level. Virulence associated genes of Indian isolates Rs-09-161 and Rs-10-244 had been studied and compared them with the isolates available globally. This involves identifying the virulence factors and T3Es present in Indian isolates and analyzing the coding sequences of these genes for generating similarity matrix and phylogenetic trees.

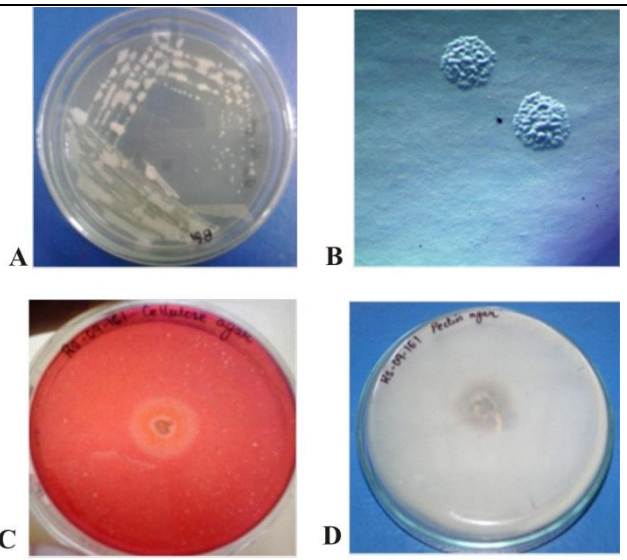


Fig. 3.1. Phenotypic depiction of virulence factors present in *R. solanacearum* isolate Rs-09-161. (A) Excessive EPS production by Rs-09-161 (B) Twitching motility under 10X magnification. (C) Cellulase production (D) Pectinase production.

The basic architecture of the *R. solanacearum* genome consists of two replicons; the Chromosome and the megaplasmid (Salanoubat *et al.*, 2002). The genome of Rs-09-161 is 5.65 Mb and Rs-10-244 is 5.66 Mb in size. The size of the chromosome is 3.68 Mb and 3.66 Mb while the megaplasmid is 1.95 Mb and 1.99 Mb in Rs-09-161 and Rs-10-244 respectively. This is consistent with the representative isolate GMI1000 where the chromosome is 3.72 Mb and the megaplasmid is 2.09 Mb (Salanoubat *et al.*, 2002) (Table 3.3).

Table 3.3 Comparison of the General features of the *R. solanacearum* isolate Rs-09-161 and Rs-10-244 with GMI1000

Features	Rs-09-161	Rs-10-244	GMI1000
Genome size (Mb)	5.645	5.659	5.81
Chromosome	3.68	3.66	3.72
Megaplasmid	1.95	1.99	2.09
Percent GC	66.82	66.98	66.97
Number of protein coding genes	5185	5171	5129
Average length of protein coding genes (bp)	974.85	969.3	989

3.3.2 Analysis of virulence factors

The virulence genes in the test isolates were identified using GMI1000 (Phylotype-I) as standard reference isolate. The details of virulence genes of *R. solanacearum* isolate Rs-09-161 and Rs-10-244 their location in the genome and the probable function are given in table 3.2. These includethe coding sequences of the genes responsible for the production of EPS, PCWDE, chemotaxis, swimming motility and twitching motility. The nomenclature of virulence genes is with the prefix RALSO161 and RALSO244 for Rs-09-161 and Rs-10-244 respectively. A similarity matrix obtained by comparing the sequences of virulence genes (EPS, PCWDE, Chemotaxis, Swimming motility and Twitching motility) of Rs-09-161 and Rs-10-244 with other reference isolates are given in table 3.4 to 3.6**.** The sequences of Rs-09-161 and Rs-10-244 share more than 99% similarity between them and are closely related to GMI1000.

The main virulence factor of *R. solanacearum*, the EPS is secreted by seven genes *viz.*, *epsA*, *epsB, epsC, epsD, epsF, epsP* and *epsR*. The gene *epsD* is absent in phylotype I isolates and all the EPS contributing genes are located on the megaplasmid. The sequences of CMR15

(phylotype III) and Psi07 (phylotype IV) share more than 90% similarity with Rs-09-161 and Rs-10-244 (Table 3.4).Both Rs-09-161 and Rs-10-244, have the presence of all six PCWDE genes.The sequences of PCWDE genes of Rs-09-161 and Rs-10-244 share 99% similarity with that of GMI1000 (Table 3.5). The only gene present on the chromosome is *pehB* and this observation is consistent with the reports of other *R. solanacearum* isolates. This shows that along with the major housekeeping genes, some of the essential virulence associated genes are also present on the chromosome (Genin and Denny, 2012).

Motility associated genes in *R. solanacearum* help the bacterium to locate and invade the host root for colonization (Meng *et al.,* 2011). The genes identified for chemotaxis (*cheA*, *cheW)* and swimming motility (*fliC, flgM*) are located on the megaplasmid whereas twitching motility (*pilA*, *pilP*) are located on the chromosome. The mutants of swimming motility are highly reduced in the degree to cause virulence on tomato plants under natural conditions (Tans-Kersten *et al.,* 2001) and of chemotaxis are completely non-chemotactic (Yao and Allen, 2006).The swimming motility and chemotaxis associated genes *fliC*, *pilP* and *cheW* are found to be highly conserved among all phylotypes and share more than 95% similarity (Table 3.6). These are probably the regions which are not evolving or are conserved across the phylotypes. Twitching motility is a trait associated with the type IV pili and plays an important role in autoaggregation and biofilm formation (Kang *et al.,* 2002). The *pilP* gene of Rs-10-244 shares 100% similarity with GMI1000 whereas Rs-09-161 is 99% similar. *PilA* gene shares 89% similarity within the two isolates and 91% with GMI1000. *PilA* exhibits diversity in the sequence among the other phylotypes of *Ralstonia solanacearum* Species Complex (*RSSC)* and is probably the region which undergoes evolution thus can be used for designing primers for isolate wise differentiation. The gene *pilA* has been used to study the

genetic diversity in soil bacterium *Myxococcus xanthus* isolates and has shown highest polymorphism in comparison to that of other genes used (Vos and Velicer, 2006).

Sequences of virulence associated genes in isolates Rs-09-161 and Rs-10-244 are found to be more close to Phylotype III and Phylotype IV isolates. Similar results were also observed by Ramesh*et al.* (2014a) when *egl* and *hrp* gene sequences from Phylotype I isolates were analyzed.

Table 3.4Sequence similarity of genes coding for EPS of Rs-09-161 and Rs-10-244 with representative phylotype isolates.

	epsA		*epsB*		*epsC*		*epsE*		*epsF*		*epsP*		*epsR*	
	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244
Rs-09-161														
Rs-10-244	1		1		1		1		1		1		1	
GMI1000	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CFBP2957	0.91	0.91	0.92	0.92	0.94	0.94	0.89	0.89	0.89	0.89	0.91	0.91	0.9	0.9
Po82	0.9	0.9	0.93	0.93	0.93	0.94	0.89	0.89	0.89	0.89	0.92	0.92	0.89	0.89
CMR15	0.96	0.95	0.98	0.97	0.97	0.97	0.98	0.98	0.96	0.96	0.98	0.98	0.97	0.97
Psi07	0.93	0.93	0.94	0.94	0.96	0.96	0.92	0.92	0.91	0.9	0.95	0.95	0.9	0.9

Table 3.5 Sequence similarity of genes coding for CWDE of Rs-09-161 and Rs-10-244 with representative phylotype isolates.

	pglA		*pehB*		*pehC*		*pme*		*egl*		*cbhA*	
	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244
Rs-09-161												
Rs-10-244	0.99		0.99		0.99		1.00		0.99		1.00	
GMI1000	0.99	0.99	0.99	0.99	0.99	1.00	1.00	1.00	0.99	1.00	0.99	0.99
CFBP2957	0.91	0.91	0.90	0.90	0.96	0.96	0.91	0.91	0.93	0.93	Absent	absent
Po82	0.90	0.90	0.90	0.90	0.92	0.93	0.91	0.91	0.93	0.93	Absent	absent
CMR15	0.95	0.95	0.97	0.97	0.97	0.97	0.95	0.95	0.95	0.95	0.88	0.88
Psi07	0.91	0.91	0.89	0.88	0.93	0.93	0.90	0.90	0.95	0.95	0.95	0.95

Table 3.6. Sequence similarity of genes coding for chemotaxis, swimming motility and twitching motility of Rs-09-161 and Rs-10-244 with representative phylotype isolates.

	cheA		*chew*		*fliC*		*flgM*		*pilA*		*pilP*	
	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244	Rs-09-161	Rs-10-244
Rs-09-161												
Rs-10-244	1.00		1.00		1.00		1.00		0.90		1.00	
GMI1000	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.91	0.93	1.00	1.00
CFBP2957	0.95	0.95	0.97	0.97	0.98	0.98	0.91	0.91	0.88	0.94	0.94	0.95
Po82	0.95	0.95	0.98	0.98	0.96	0.96	0.90	0.90	0.82	0.79	0.94	0.94
CMR15	0.98	0.98	0.99	0.99	1.00	1.00	0.95	0.96	0.74	0.72	0.98	0.99
Psi07	0.95	0.95	0.97	0.97	0.95	0.95	0.95	0.95	0.75	0.75	0.96	0.97

3.3.3 Identification of T3Es

Seventy two T3Es in Rs-09-161 and 77 T3Es in Rs-10-244 (including one multiple copy T3E) were identified based on the identification criteria described. The identified T3Es are assigned the names with prefix Rip (Ralstonia Injected Protein) as per the newly proposed nomenclature by Peeters and co-workers (2013) and the locus tag of the T3Es is represented by the prefix 161_ and 244_ for Rs-09-161 and Rs-10-244 respectively. The T3E gene *RipTPS* is present in multiple copies in both the isolates. Rs-09-161 has the presence of three candidate effectors (*Rs_T3E_Hyp6, Rs_T3E_Hyp7* and *Rs_T3E_Hyp15*) and three pseudogenes (*RipF1, RipAX1* and *Rs_T3E_Hyp8*). Rs-10-244 has the presence of one candidate effectors (Rs_T3E_Hyp7) and four pseudogenes (*RipO1, RipAX2, Rs_T3E_Hyp8* and *Rs_T3E_Hyp15*). Pseudogenes are non-functional genes and its presence can be attributed to the fact that either these genes are been mutated due to the presence of transposable elements within the gene thus leading to its disruption or due to errors in sequencing (Table 3.7).

Table 3.7 Identification of T3Es in *R. solanacearum* isolates Rs-09-161 and Rs-10-244

T3E Gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RipA1	RSc2139	161_20690	68.36	3177	99	244_36120	68.32	3204	99
RipA2	RSp0099	161_34090	70.3	3378	99	244_00720	70.44	3384	99
RipA3	RSp0846	161_40770	71.96	3702	99	161_40780	71.96	3717	99
RipA4	RSp0847	161_40790	72.43	3990	99	244_07960	72.5	3990	99
RipA5	RSP1024	161_42360	69.51	2723	99	244_09460	69.43	3726	99
RipB	RSc0245	161_02320	69.25	1584	92	244_18450	69.1	1479	99
RipC1	RSp1239	161_44460	66.87	2832	99	244_11640	66.41	2745	99
RipC2	RCFBP_mp20032	Nil	0	0	0	244_24680	59.83	2490	81
RipD	RSp0304	161_35920	63.61	1932	99	244_02680	63.71	1932	99
RipE1	RSc3369	161_31680	67.84	1278	97	244_47820	68.38	1278	99
RipE2	RCFBP_mp10565	Nil	0	0	0	244_24320	57.64	909	88
RipF1	RSp1555	Pseudogene	0	0	0	244_08360	64.41	2181	99
RipF2	RCFBP_mp30453	Nil	0	0	0	Nil	0	0	0
RipG1	RSp0914	161_41320	65.7	1986	99	244_08450	65.71	1998	99
RipG2	RSp0672	161_39180	69.35	3129	98	244_06280	69.41	3129	99
RipG3	RSp0028	161_33870	68.28	1797	98	244_00270	68.06	1794	99
RipG4	RSc1800	161_17570	71.34	1389	99	244_33040	71.34	1389	99
RipG5	RSc1801	161_17580	69.88	1617	99	244_33050	70.19	1617	99
RipG6	RSc1356	161_13550	68.85	1875	88	244_29420	68.75	1866	88

T3E gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RipG7	RSc1357	161_13560	64.97	1836	80	244_29430	66.89	1935	98
RipG8	CMR15v4_10224	Nil	0	0	0	Nil	0	0	0
RipH1	RSc1386	161_13830	69.3	2304	99	244_29700	69.32	2298	99
RipH2	RSp0215	161_35090	67.76	2274	98	244_01730	68.28	2229	98
RipH3	RSp0160	161_34560	67.54	2160	99	244_01190	67.68	2160	99
RipH4	RPSI07_mp0161	Nil	0	0	0	Nil	0	0	0
RipI	RSc0041	161_00420	65.5	1206	99	244_16460	64.73	1293	99
RipJ	RSc2132	161_41350	57.41	2712	78	244_36060	58.87	1296	99
RipK	RCFBP_mp10024	Nil	0	0	0	Nil	0	0	0
RipL	RSp0193	161_34900	69.03	4176	98	244_01530	69.06	4173	99
RipM	RSc1475	161_18160	71.5	1755	99	244_33610	71.5	1755	99
RipN	RSp1136	161_43460	66.1	1425	99	244_10610	66.03	1425	99
RipO1	RSp0323	161_36140	61.2	1539	98	Pseudogene	62.36	1047	99
RipO2	RALSY_mp30159	Nil	0	0	0	Nil	0	0	0
RipP1	RSp0826	Nil	0	0	0	244_24210	55.46	1107	100
RipP2	RSp0868	161_08570	59.71	1467	99	244_24510	59.71	1467	99
RipP3	RSY45_33690	Nil	0	0	0	244_46550	61.41	1161	99
RipQ	RSp1277	161_44830	68.85	1557	99	244_12020	69.04	1557	99
RipR	RSp1281	161_44870	70.6	5229	99	244_12060	70.66	5229	99
RipS1	RSc3401	161_32000	69.04	7056	98	Nil	0	0	0
RipS2	RSp1374	161_45440	70.18	7539	99	244_12410	69.99	7458	99

T3E gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RipS3	RSp0930	161_41420	66.81	6876	99	244_08550	66.85	6876	99
RipS4	RSc1839	161_17940	70.78	7725	99	244_33400	70.78	7725	99
RipS5	RSp0296	161_35870	67.55	7017	99	244_02630	67.5	7017	99
RipS6	RSc2130	161_20590	66.62	2424	99	244_36050	66.35	2547	99
RipS7	RPSI07_mp1267	Nil	0	0	0	Nil	0	0	0
RipS8	RPSI07_1850	161_17980	68.77	9303	92	244_3343	68.89	9294	92
RipT	RSc3212	Nil	0	0	0	244_46320	56.62	966	99
RipU	RSp1212	161_44210	62.57	879	0	244_11370	61.84	891	100
RipV1	RSc1349	161_13470	67.95	2013	100	244_29350	68	2013	99
RipV2	RPSI07_1895	Nil	0	0	0	Nil	0	0	0
RipW	RSc2775	161_26420	63.61	1146	95	244_41770	63.59	1140	99
RipX	RSp0877	161_41090	66.76	996	95	244_08260	67.05	1023	98
RipY	RSc0257	161_02390	67.2	2790	99	244_18510	65.36	2844	72
RipZ	RSp1031	161_42440	69.22	4110	99	244_09550	69.12	4113	99
RipAA	RSPI07_2742	161_06050	62.56	804	87	244_22050	62.4	798	87
RipAB	RSp0876	161_41080	65.14	525	99	244_08250	64.76	525	99
RipAC	RSp0875	161_41070	63.59	2958	97	244_08240	62.92	2643	99
RipAD	RSp1601	161_47560	64.11	978	98	244_14660	64.21	978	99
RipAE	RSc0321	161_03080	66.4	1932	99	244_19150	67.02	1932	99
RipAF1	RSp0822	0161_4053	1020	846	95	244_07700	68.78	1041	97
RipAF2	RALSY_20037	Nil	0	0	0	Nil	0	0	0

T3E gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RipAG	RSc0824	161_08850	59.59	297	98	244_24200	60.6	297	100
RipAH	RSc0895	Nil	0	0	0	244_24720	62.88	291	100
RipAI	RSp0838	161_40690	70.75	612	99	244_07860	71.07	612	99
RipAJ	RSc2101	161_20300	69.15	1005	99	244_35750	69.05	1005	99
RipAK	RSc2359	Nil	0	0	0	244_38200	65.72	2430	99
RipAL	RPSI07_mp0618	161_39680	70	1350	99	244_06790	70	1350	99
RipAM	RSc3272	161_30700	71.82	465	99	244_46830	71.18	465	99
RipAN	RSp0845	161_40760	67.96	4323	99	244_07940	67.82	4323	99
RipAO	RSp0879	161_41110	68.96	1479	97	244_08280	69.57	1479	98
RipAP	CMR15v4_10224	161_44240	67.32	2863	93	244_11400	67.49	2421	93
RipAQ	RSp0885	161_41170	69.6	2112	99	244_08340	69.5	2112	99
RipAR	RSp1236	161_44430	69.53	1815	96	244_11600	69.63	1818	98
RipAS	RSp1384	161_45560	71.86	2802	99	244_12510	71.73	2802	99
RipAT	RSp1388	161_45600	70.65	1755	99	244_12550	70.59	1755	99
RipAU	RSp1460	161_46300	67.19	939	99	244_13250	67.3	939	99
RipAV	RSp0732	161_39740	68.3	2538	99	244_06850	68.7	2538	99
RipAW	RSp1475	161_46450	66.29	1347	99	244_13400	66.44	1347	97
RipAX1	RSc3290	Pseudogene	0	0	0	244_47020	58.68	759	100
RipAX2	RSp0572	161_38220	57.99	657	99	Pseudogene	0	0	0
RipAY	RSp1022	161_42340	64.48	1236	97	244_09440	64.56	1236	97
RipAZ1	RSp1582	161_47400	61.03	834	99	244_14480	60.91	834	99

T3E gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RipAZ2	RALSY_20407	Nil	0	0	0	Nil	0	0	0
RipBA	RSc0227	161_02140	54.71	594	99	244_18270	55.21	594	98
RipBB	RPSI07_mp0573	Nil	0	0	0	244_05100	61.43	1320	0
RipBC	RCFB_mp30170	Nil	0	0	0	Nil	0	0	0
RipBD	RALSY_20184	Nil	0	0	0	Nil	0	0	0
RipBE	RS1000_RIP10	Nil	0	0	0	Nil	0	0	0
RipBF	RPSI07_2863	Nil	0	0	0	Nil	0	0	0
RipBG	RSMK00763	Nil	0	0	0	Nil	0	0	0
RipBH	RPSI07_mp30113	Nil	0	0	0	Nil	0	0	0
RipBI	RCFB_mp30113	Nil	0	0	0	Nil	0	0	0
RipTAL	RSc1815	161_17720	66.04	3726	99	244_33180	66.05	3738	99
RipTPS	RSp0731	161_39730 161_43110	68.68	1785	99	244_06840 244_10270	68.57	1785	99
RS_T3E_Hyp1	RSPsi07_0331	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp2	RSPsi07_1883	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp3	RSPsi07_mp0834	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp4	RSPsi07_mp1047	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp5	RSPsi07_mp1559	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp6	CMR15v4_30001	161_32360	55.45	1311	88	Nil	0	0	0

T3E gene	reference LT*	Locus tag Rs-09-161	GC%	length	%blast	Locus tag Rs-10-244	GC%	length	%blast
RS_T3E_Hyp7	RSMK06225	161_32180	55.1	519	0	244_48280	55.1	519	0
RS_T3E_Hyp8	RSMK02655	Pseudogene	0	0	0	Pseudogene	0	0	0
RS_T3E_Hyp9	RRSL_01783	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp10	RSMK02638	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp11	RSMK01187	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp12	RSMK03335	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp13	RSPO_m01098	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp14	BDB mp_40006	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp15	RSPsi07_1860	161_35100	52.73	2799	94	Pseudogene	0	0	0
RS_T3E_Hyp16	RSc3174	Nil	0	0	0	Nil	0	0	0

Locus tag of representative isolates given in the above table: (RSc/RSp: GMI1000), (RCFBP: CFBP2957), (CMR15: CMR15), (RPSI07: Psi07), (RSMK: Molk2), (BDB: BDB R229), (RSPO: Po82), (RALSY: R24), (RSY45: Y45)

Comparison of the functional T3Es genes of *R. solanacearum* isolates Rs-09-161, Rs-10-244 and GMI1000 revealed 60 common T3Es within the three isolates. Rs-09-161 has the presence of two unique T3Es (*Rs_T3E_Hyp6* and *Rs_T3E_Hyp15*) and shares 63 common effectors with GMI1000 and 66 T3Es with Rs-10-244. Rs-10-244 bears four unique T3Es (*RipC2, RipE2, RipP3* and *RipBB*) and shares 66 common effectors with GMI1000 (Fig. 3.2).

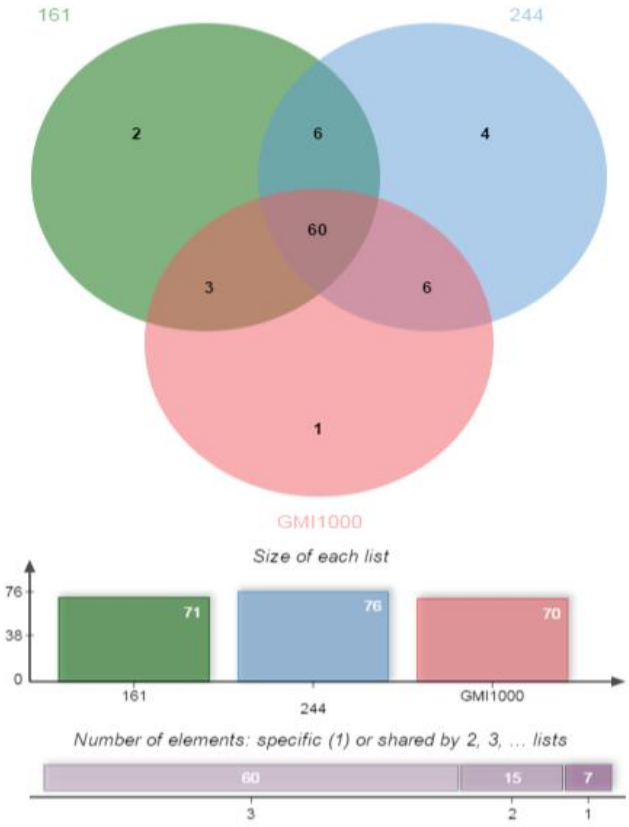


Fig. 3.2. Venn diagram constructed using T3Es complete genes of *R. solanacearum* isolate Rs-09-161(green), Rs-10-244(blue) and GMI1000(pink). The merged regions display the T3Es shared between the isolates. In Rs-09-161 and Rs-10-244, one effector is present in multiple copies which is not indicated in the bar graph.

Majority of the *R. solanacearum* isolates have an average of 70-75 T3E, which is much larger than many other bacterial plant pathogens like *P. syringe* and *Xanthomonas sp.* wherein it is in the range of 30-40 (Hajri *et al.,* 2011; Zumaquero *et al.,* 2010). Hence, it is presumed that an ancestor of *R. solanacearum* probably possessed a large number of effectors because the majority of the isolates possess a high number of effectors. The only exception to this is BDB and has less number of effectors (Genin and Denny, 2012).

Many of the *R. solanacearum* T3Es have been validated through translocation studies (Sole *et al.,* 2012; Cunnac *et al.,* 2004b; Mukaihara *et al.,*2004; Mukaihara and Tamura, 2009; Mukaihara *et al.,*2010). Many of the effectors share homology with those of other bacterial plant pathogens like *Pseudomonas syringae, Xanthomonas sp*. and *Acidovorax sp*.

Few of these effectors are present as effector families and have three to eight effectors present. These include RipA (AWR family), RipG *(GALA* family*),* RipH (*HLK* family) and the RipS (*SKWP* family) (Mukaihara *et al.,* 2010; Poueymiro and Genin, 2009; Remigi *et al.,* 2011; Sole *et al.,* 2012; Peeters *et al.,* 2013). These effectors possess certain inherent characters about the sequence such as specific internal repeats within them, which characterizes them to constitute a family. The T3SS secretes T3Es in a highly specialized manner in the eukaryotic hosts and is used by many plant and animal pathogenic bacteria, as a tool to colonize in their respective hosts.

The various motifs or domains present on the T3Es interact with the host cells and benefits the bacteria in colonization. The T3Es are secreted into the cytosol of the host through the *hrp* pili. Homology search in NCBI conserved domains for the unique T3Es of *R. solanacearum* isolate Rs-09-161 and Rs-10-244 revealed the presence of various motifs. The T3E *RipBB* present in Rs-10-244 exhibits presence of ankyrin repeats which mediate protein-protein interactions in diverse families of proteins. *RipC2* shares homology with

Haloacid dehalogenase (HAD)-like hydrolases and *RipP3* which is also known as *PopP3* displays YopJ Serine/Threonine acetyltransferase activity. The T3E *Rs_T3E_Hyp15* present in Rs-09161 displays presence of Serine/Threonine protein kinase domain within it.

3.3.4 Analysis of T3Es

3.3.4.1 Rip A family

RipA (*AWR* family) effectors include five effectors (*RipA1* to *RipA5*), and both the isolates, Rs-09-161 and Rs-10-244 have all the RipA effectors present in them. Among the RipA effectors, *RipA1* is present only in Phylotype I isolates, whereas *RipA2* along with *RipA4* and *RipA5* is present in all the phylotypes of *R. solanacearum* isolates studied till date. *RipA5* is also present in multiple copies in some of the phylotype II isolate (Molk2, IPO1609, UW551 and Po82). The phylogenetic tree constructed for *RipA* gene family is depicted in Fig. 3.3. The isolates have grouped together based on their phylotypes. Rs-09-161 and Rs-10-244 are present in the group formed by phylotype I isolates. The group formed by phylotype I isolates is closely related to the phylotype III group formed by CMR15. Phylotype II and phylotype IV have separated to form two different groups. The RipA effectors consist of a conserved region containing the alanine-tryptophan-arginine tryad and can be virulent or avirulent depending on the host with which *R. solanacearum* interacts. *RipA2* was found to be a major contributor to the virulence among the AWR family (Sole *et al.*, 2012). Different plant pathogenic isolates like *Burkholderia glumae*, *Acidovorax avenae* and animal pathogenic isolate like *B. pseudomallei* have the presence of AWR effectors (Mole *et al.*, 2007; Viallard *et al.*, 1998; Willems *et al.*, 1992; Wuthiekanun and Peacock, 2006).

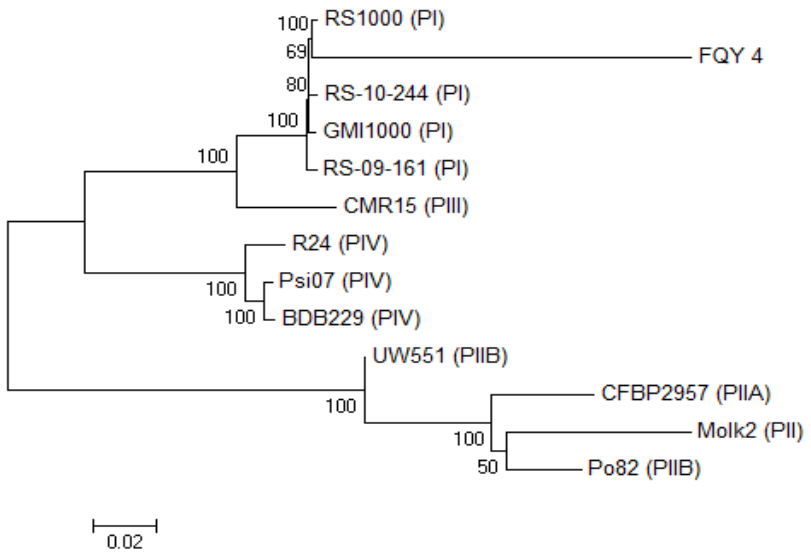


Fig.3.3 Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipA (AWR family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in brackets. The tree was generated by MEGA-6 (Tamura *et al.*, 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap re-samplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

3.3.4.2 Rip G family

RipG (*GALA* family) possesses eight T3Es (*RipG1* to *RipG8*) and seven (*RipG1* to *RipG7*) are present in both Rs-09-161 and Rs-10-244. The phylogenetic tree constructed for the RipG gene family is depicted in Fig. 3.4. The phylotype IV isolate R24 has separated to form a completely different group. The phylotype I isolates have grouped together in a single cluster and are closely related to the phylotype III isolate CMR15. Rs-09-161 and Rs-10-244 are present in the phylotype I group. The phylotype II isolates have formed a single group and phylotype IV isolates; Psi07 and BDBR229 have formed a different group respectively. The RipG has the presence of leucine rich repeats (LRR) and F-box domain with them. The F-box protein forms a component of E3-Ubiquitin ligase complex and is found in eukaryotes. This complex plays an important role in ubiquitination of proteins which leads to the degradation or modification of the activity of the targeted

protein (Hua and Vierstra, 2011). *RipG8* is present only in CMR15 (phylotype III). More isolates from Phylotype III needs to be studied to identify if the RipS8 is specific to phylotype III isolates.

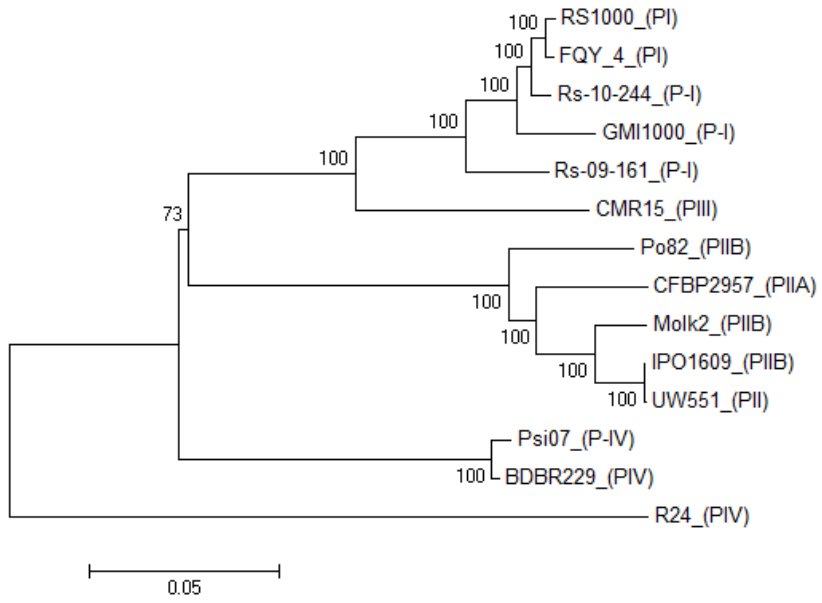


Fig.3.4 Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipG (GALA family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in brackets. The tree was generated by MEGA-6 (Tamura *et al.*, 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap re-samplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

3.3.4.3 Rip H family

RipH (*HLK* family) consists of four effectors (*RipH1* to *RipH4*); Rs-09-161 and Rs-10-244 has the presence of *RipH1*, *RipH2* and *RipH3* with an average size of ~600 amino acids. *RipH4* is found to be present only among phylotype IV isolates. The phylogenetic tree constructed for *RipH* gene family is shown in Fig. 3.5. The isolates have grouped together based on their phylotypes. Phylotype I isolates have formed a single group and are closely associated with the group formed by phylotype III isolate CMR15. Two different groups

were formed by phylotype II and phylotype IV isolates. The RipH family is named so because of the presence of histidine-leucine–lysine triad in a conserved C-terminal region. Phylogenetic analysis of the RipH effectors indicates an ancestral isolate of *R. solanacearum* most likely had only three RipH effectors and the fourth one has evolved later independently (Chen *et al.*, 2014).

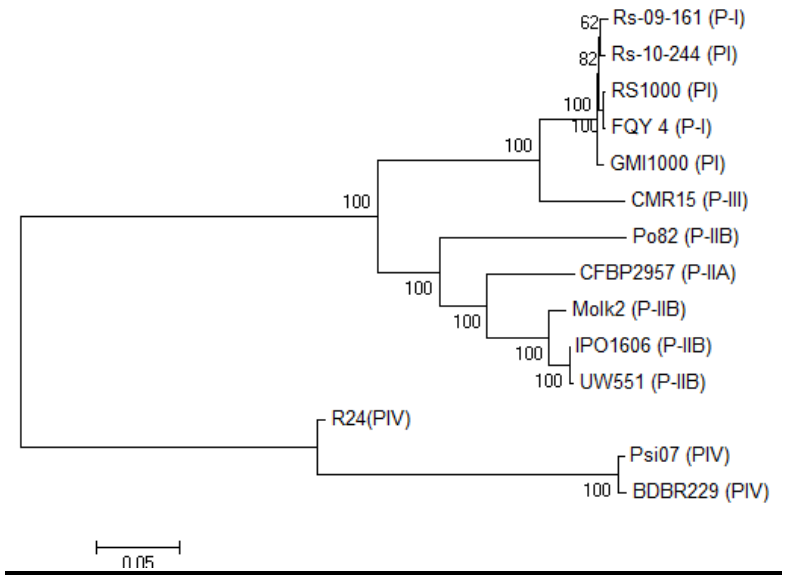


Fig.3.5. Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipH (HLK family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in brackets. The tree was generated by MEGA-6 (Tamura *et al.*, 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap re-samplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

3.3.4.4 Rip S family

RipS (*SKWP* family) has eight effectors (*RipS1*to *RipS8*); RipS7 is absent in Rs-09-161 whereas RipS1 and RipS7 are absent in Rs-10-244. *RipS7* is absent in all Phylotype I isolates studied till date and is present in all phylotype IV isolates. *RipS1* and *RipS6* is absent in all phylotype IV isolates. Phylotype II lacks *RipS6* and *RipS8*. The phylogenetic tree constructed for RipS family is shown is Fig. 3.6. Phylotype I isolates have grouped

together and are closely related to the group formed by phylotype III isolate CMR15. Phylotype IV isolates have separated to form two groups. Phylotype II isolates have split into two different groups. The structure of RipS (SKWP family) effectors is found to be related to Heat/armadillo repeat domain. The RipS proteins exert their virulence on their host plant by interaction through the SKWP domain (Mukaihara and Tamura, 2009).

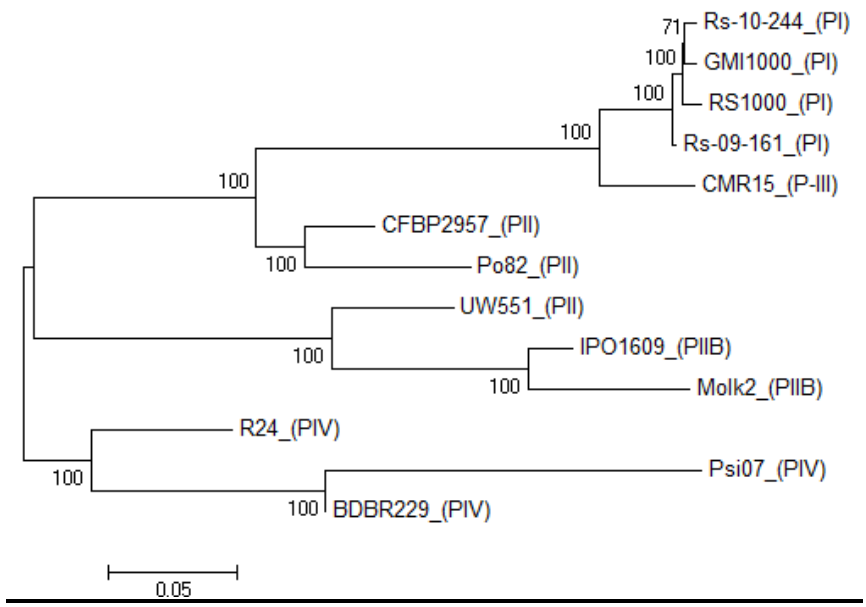


Fig.3.6. Phylogenetic tree constructed using neighbour-joining method based on the coding sequences of effectors of RipS (SKWP family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in brackets. The tree was generated by MEGA-6 (Tamura *et al.*, 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1,000 bootstrap re-samplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

3.4 Conclusion

Analysis of the virulence genes and the T3E genes of *R. solanacearum* isolates Rs-09-161 and Rs-10-244 indicated that a majority of the virulence associated genes are present in both the isolates. It was observed that all the virulence genes of Rs-09-161 and Rs-10-244 are highly conserved and share high level of similarity except for *pilA* gene, which shares a minimum of 72% similarity. Seventy two T3E genes were identified in *R. solanacearum*

isolate Rs-09-161 and 77 in Rs-10-244. Phylogenetic analysis of T3E genes of *RipA, RipG, RipH and RipS* revealed grouping of the isolates based on their phylotypes. Group 1 consists of isolates that belong to phylotype I including Rs-09-161 and Rs-10-244. Phylotype III isolate CMR15 forms a group closely associated with Phylotype I. The isolates belonging to phylotype II (IIA and IIB) and phylotype IV have separated to form two different groups. This grouping is consistent with the four gene families studied here. Similar results are also observed by Peeters *et al.* (2013) and Remenant *et al.* (2011), wherein isolates from phylotype I and III have clustered together. It is likely that the isolates from phylotype I and phylotype III did not undergo much evolution and hence form a major group (Remenant *et al.*, 2011).

CHAPTER IV

Development of mutants of *R. solanacearum* lacking important virulence factors

4.1. Introduction

Ralstonia solanacearum is regarded as an important soil borne phytopathogen with wide host range and larger geographical distribution (Genin and Denny, 2012). The virulence of this pathogen is contributed by various virulence and pathogenicity factors. These include Exopolysaccharide (EPS), the type II secretory system (T2SS) dependent plant cell wall degrading enzymes, chemotaxis, swimming motility, twitching motility and type III secretory system (T3SS) (Genin and Denny, 2012). The type six secretion system (T6SS) has been recently identified in *R. solanacearum* and contributes towards the virulence (Zhang *et al.,* 2012).

The T3SS plays a crucial role in the pathogenicity of *R. solanacearum*. It is encoded by the *hrp* (hypersensitive response and pathogenicity) regulon and defects in the T3SS impair the induction of hypersensitive response in resistant hosts and pathogenicity in its susceptible hosts (Genin and Denny, 2012). The T3SS injects pathogenicity proteins termed as Type III effectors (T3Es) into the eukaryotic hosts through the *hrp* pili (Mukaihara and Tamura, 2009). These effectors are directly translocated into the cytosol of the hosts (Poueymiro and Genin, 2009).

Once within the host cells, the effectors promote its colonisation by interacting with various host proteins and subverting the host immunity (Poueymiro *et al.,* 2014). The T3SS and the expression of T3Es is controlled by an AraC family transcriptional regulator called as the *hrpB* (Genin and Denny, 2012; Coll and Valls, 2013). Recently 72 and 77 T3Es were reported in *R. solanacearum* isolates Rs-09-161 and Rs-10-244 (Asolkar and Ramesh, 2018a). T3Es expressed by *R. solanacearum* are validated through translocation studies in GMI1000 and RS1000 with the help of T3SS mutants (Mukaihara and Tamura, 2009; Mukaihara *et al.,* 2010; Sole *et al.,* 2012). Many T3E are reported as putative T3Es

and are not yet validated through translocation studies. Validation of these T3Es will help in understanding the mechanism of pathogenesis in this bacterium.

In addition to T3SS, other secretion systems like T4SS, T5SS and T6SS are also present in bacteria. The T6SS is a recently discovered secretion system (Pukatzki *et al.,* 2006). Since the discovery of the T6SS is quite recent, limited research is available on this secretion system. The T6 secretion gene cluster is widely present in the genome of many gram negative bacteria, especially proteobacteria (Boyer *et al.,* 2009; Cascales and Cambillau, 2012) and is involved in pathogenesis, bacterial interactions and competition. The T6SS interacts with both prokaryotic and eukaryotic cells (Silverman *et al.,* 2012). The T6SS secretes anti-bacterial proteins into the target cells and thus kills the neighbouring, non-immune bacterial cells by cell-to-cell contact (Cascales and Cambillau, 2017).

The T6SS is also involved in translocation of substrates into the recipient cells through contact dependent manner (Silverman *et al.,* 2012; Cornelis *et al.,* 2006). In eukaryotes, it secretes toxin molecules which interfere with the eukaryotic cytoskeleton and thus plays a role in pathogenesis (Cascales and Cambillau, 2012). In *R. solanacearum,* the T6SS genes *tssM* and *tssB* have already been proved to be involved in virulence. Mutation of these genes has led to the attenuation of virulence on tomato plants in comparison to the wild type isolate (Zhang *et al.,* 2012; 2014). However, the other genes have not been studied for their role in pathogenesis or virulence.

In case of T3SS, mutagenesis of *hrpB* gene by insertion of Ω interposons has proved it to be the positive regulator of the system (Genin *et al.,* 1992). Mutants of *hrpB* and *hrpG* developed by Ω interposons insertion have shown critically less colonisation in the susceptible hosts during different stages of infection (Vasse *et al.,* 2000). The *hrpB* and *hrcV* mutants are used in the validation of the T3E in *R. solanacearum*(Mukaihara *et*

al.,2009; Cunnac *et al.,* 2004b). The *hrcV* is a secretion deficient mutant of T3SS. The presence of a T3E protein in wild type and its absence in *hrpB, hrpG* or *hrcV* mutant can validate a putative T3E.

In order to validate a novel T3E and to identify the importance of T6SS in virulence of *R. solanacearum*, this study was taken up with the objective "Development of mutants of *R. solanacearum* lacking important virulence factors".

4.2. Materials and methods

4.2.1. *R. solanacearum* isolate

*R. solanacearum*isolate Rs-09-161 maintained at the culture collection of Plant Pathology laboratory, ICAR-CCARI was used as the parent isolate in this study. The isolate was maintained as 30% glycerol stock at -80°C. *R. solanacearum*was revived on BG agar supplemented with TZC and subcultured on BG medium (Appendix II) at 28°C for 48 h.

4.2.2. PCR amplification of internal fragments of gene

4.2.2.1. Selection of genes

Seven different genes were selected in *R. solanacearum* isolate Rs-09-161 for development of mutants. These included two genes *viz., hrpB* and *hrcV* which belong to the T3SS and five genes *viz., tssM, tssH, hcp, vgrG3* and o*mpA* genes that belong to the T6SS. The details about the genes are given in the table 4.1.

Table 4.1. List of genes used in the study, details about the locus tag, size and location.

Gene	Locus tag in Rs-09-161	Locus tag in standard isolate	Location	Size of the gene (bp)
		T3SS		
hrpB	RALSO161_m00208530	RSp0873	Megaplasmid	1434
hrcV	RALSO161_m00208430	Rsp0863	Megaplasmid	2073
		T6SS		
tssM	RALSO161_m00207390	RSp0763	Megaplasmid	3930
tssH	RALSO161_m00207270	Rsp0749	Megaplasmid	2718
hcp	RALSO161_m00207230	Rsp0745	Megaplasmid	504
vgrG3	RALSO161_m00207440	Rsp0768	Megaplasmid	1605
ompA	RALSO161_m00207370	RSPOc02457	Megaplasmid	840

Locus tag: RSp-GMI1000, RSPO-Po82

4.2.2.2. Isolation of genomic DNA

Isolation of genomic DNA was performed as mentioned by Ramesh *et al*. (2014a). The reagents used for DNA isolation are given in appendix III. DNA isolation was carried out using overnight grown cells of Rs-09-161 maintained at 150 rpm at room temperature (RT). The culture (1.5 mL) was centrifuged at 10000 rpm for 10 min and the supernatant was discarded. The cell pellet was resuspended in 600 µL of lysis buffer, mixed thoroughly and incubated at 37°C for 1 h. To this 100 µL of 5 M NaCl was added, mixed thoroughly, followed by 80 µL of CTAB/NaCl solution and incubation at 65°C for 10 min. Equal amount of chloroform/isoamyl alcohol was added, mixed thoroughly and centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred to a fresh tube and equal amount of phenol/chloroform/isoamyl alcohol was added. The tubes were mixed thoroughly and centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred to a fresh tube and the nucleic acids were precipitated in 0.6 volume of isopropanol. The

tubes were mixed thoroughly and centrifuged at 10000 rpm for 10 min. The precipitated DNA was washed with 70% ethanol, dried and the pellet was re-dissolved in 50µL of TE buffer. The DNA was quantified using Nano drop-1000, Thermo fisher scientific, USA and maintained at -30°C for subsequent use. The DNA was diluted to 50 ng/µL and used for subsequent PCR amplification and agarose gel electrophoresis.

4.2.2.3. Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel electrophoresis apparatus (Tarson Products Pvt. Ltd., India) as per manufacturer's instructions. Agarose gel (0.8%) was casted using 0.5 $\mu g.mL^{-1}$ of ethidium bromide in 1 X TAE buffer. DNA sample (5 µL) was mixed with 1 µL of 6 X DNA loading buffer (Thermo Scientific, USA) and loaded in the agarose gel submerged in 1 X TAE buffer. Electrophoresis was performed at 80 V and the DNA bands were visualized and documented with MultiImage Light Cabinet (Alpha Innotech Corporation).

4.2.2.4. PCR amplification of fragments

Primers were designed for the amplification of internal fragments at the 5'-end of the genes using Oligo primer analysis software. The sequences of the primers are given in table 4.2. The reaction mix and standardised PCR conditions for each gene are given in table 4.3. PCR amplification was carried out using Taq DNA polymerase which gives 3'-dA overhangs on both sides of the amplicon. All the amplifications were performed in Mastercycler Pro (Eppendorf, GmBH). The amplicons were visualised on 1.0% agarose gel containing 0.5 $\mu g.mL^{-1}$ of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The amplified products were purified using GeneJETTM PCR Purification Kit (Fermentas Life Sciences, EU) as per manufacturer's

instructions and quantified using Nano drop-1000, Thermo fisher scientific, USA. The purified products were maintained at -35°C until use.

Table 4.2. List of primers used for the amplification of internal fragment of genes

Gene	Primer	Primer sequence (5'-3')	Amplicon (bp)
		T3SS	
hrpB	HrpB-F	CGAGGAAAGTCCGACGACTA	582
	HrpB-R	CGCAGCAGGCTGAGGTATTC	
hrcV	HrcV-F	GCTGATGGTGCTGCCGCTGCC	763
	HrcV-R	GGACAGCTGCCGCACGATCTC	
		T6SS	
tssM	TssM-F	GCGATGCGGCCCTCTATGAGC	672
	TssM-R	AGGCGAACTCCAGCGGAAAGC	
tssH	TssH-F	CGTGGCTGATCGCCTCGCTGG	694
	TssH-R	TTGAGCAGATTGGCCGCATCG	
hcp	Hcp-F	TCGACAGCCCGGCCATCAAGG	421
	Hcp-R	TGGCCACCGGCGCTCTTCTGC	
vgrG3	Vgr3-F	GAGCTGTTCGAATGGCGGGTG	719
	Vgr3-R	CGGGCTGATCGACAGATAGGC	
ompA	OmpA-F	CAGGTACGGCTTCCGGCCTGC	566
	OmpA-R	CGGCCATTTCATCCAGGATGC	

F-forward primer, R-reverse primer. The same primers were also used for confirmation of insert in *E.coli*.

Table 4.3.Reaction mixture and PCR conditions for amplification of internal fragment of gene

Gene	Reaction mixture	PCR conditions
	T3SS	
hrpB	20 µL reaction mixture contained 1.0 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 10% DMSO, 20 µg BSA, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 30 s and final extension of 10 min at 72°C.
hrcV	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 5% DMSO, 20 µg BSA, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 1 min 15 s and final extension of 10min at 72°C.
	T6SS	
tssM	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing and extension at 68°C for 45 s and final extension for 10 min at 72°C.
tssH	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing and extension at 70°C for 1 min 15 s and final extension of 10 min at 72°C.
hcp	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1X assay buffer, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing and extension at 69°C for 65 s and final extension for 10 min at 72°C.
vgrG3	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing and extension at 70°C for 1 min 15 s and final extension of 10 min at 72°C.
ompA	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1X assay buffer, 3% DMSO, 20 µg BSA, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 69°C for 30 s, extension at 72°C for 40 s and final extension of 10min at 72°C.

4.2.3. Cloning of amplified product in plasmid vector

The amplified PCR product was cloned using Thermo Scientific InsTAclone PCR Cloning Kit (Cat. No. K1213). The procedure was carried out as per manufacturer's instruction. The linearized vector pTZ57R/T was used for cloning. The linearized vector pTZ57R/T has 3'-thymine overhangs within the MCS present in *lacZα* fragment of β-galactosidase. The *lacZα* fragment is flanked by M13 primers.

4.2.3.1. Ligation:

Ligation reaction was set up as follows:

Table 4.4. Ligation mixture for cloning pTZ57R/T vector

	Volume (μL)	
Component	Amplified PCR fragment	Control DNA fragment
Vector pTZ57R/T, (0.17 pmol ends)	3 μL	3 μL
5X Ligation Buffer	6 μL	6 μL
PCR product (0.52 pmol ends)	variable*	4 μL
Water, nuclease-free	to 29 μL	16 μL
T4 DNA Ligase	1 μL	1 μL
Total volume	30 μL	30 μL

The ligation reaction mix was vortexed briefly, centrifuged for 3-5 s and incubated at 22°C for 1 h. The insert:vector molar ratio was maintained as 3:1. The PCR product (0.52 pmol ends), was used with 0.165 μg (3 μL, 0.172 pmol ends) of the pTZ57R/T vector. After 1 h, 2.5 μL of the ligation mixture was used for bacterial transformation. The concentration of insert fragment (0.52 pmol ends) used for ligation is given in table 4.5.

Table 4.5.: Concentration of insert fragment (0.52 pmol ends) used in ligation.

Gene	Fragment size (bp)	Concentration (ng/µL)
hrpB	582	100
hrcV	763	131
tssM	672	115
tssH	694	119
Hcp	421	72
vgrG3	719	123
ompA	566	97

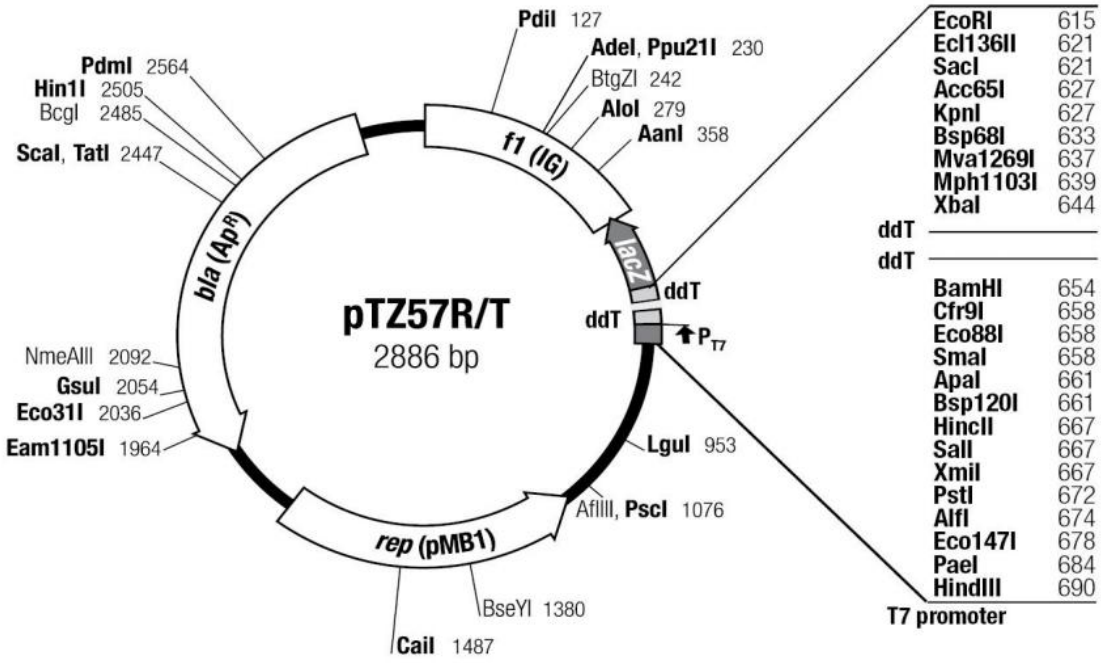


Fig. 4.1. Vector map of linearized vector pTZ57R/T

4.2.3.2. Transformation

Transformation was carried out as per manufacturer's instruction. Transformation procedure is given in brief as follows:

E.coli isolate JM107 grown at 37^{o}C was used for transformation. A portion of overnight grown bacterial culture was inoculated into two 1.5 mL of pre-warmed C-medium (present in the kit) using an inoculating loop and incubated at 37°C for 2 h on a shaker. After 2 h, the cells were pelleted by centrifugation at 3000 rpm for a min and the supernatant was discarded. Cells were resuspended in 300µL of T-solution each and incubated on ice for 5 min (T solution was prepared by mixing equal volume of T solution A and T solution B and maintained on ice). After 5 min, the cells were pelleted by centrifugation at 3000rpm for a min. The pellet was re-dissolved in 120 µL of T solution and maintained on ice for 5 mins. The cells (50 µL) were transformed by adding 2.5 µL of ligation mixture and incubated on ice for 2 mins. Transformed cells were diluted to 100 µL with LB medium and plated immediately on pre-warmed LB-ampicillin (100 µg.mL^{-1}) X-Gal/IPTGagar plates. The plates were maintained at 37^{o}C overnight.

4.2.3.3. Analysis of recombinant clones

White colonies obtained on the test plate were analysed for the presence of insert DNA using the following methods.

4.2.3.3.1. Colony PCR

Positive clones were identified from the plate and subcultured on LB-ampicillin X-Gal/IPTGagar plates. After 24 h, the colonies were confirmed for the presence of insert by colony PCR. The primers and PCR conditions were same as mentioned for amplification of the internal fragment (Table 4.2; 4.3.). The recombined cells were used as a template instead of template DNA. All amplifications were performed in Mastercycler Pro (Eppendorf, GmBH). The amplicons were visualised on 1.0% agarose gel containing 0.5 µg.mL^{-1} of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation).

4.2.3.3.2. Restriction digestion

A transformed *E.coli* colony confirmed with colony PCR was grown in LB broth containing 100 µg.mL^{-1} ampicilin overnight. The plasmid was isolated using GeneJET Plasmid Miniprep kit (#K0502, Thermo Scientific, USA) as per the manufacturer's instructions. The eluted plasmid was quantified using Nano drop-1000, Thermo fisher scientific, USA and used for restriction digestion for confirmation of the recombination. The restriction profile was developed using NEBCutter version 2.0 (Vincze *et al.,* 2003). The sequence of the recombined vector containing internal fragment cloned in the MCS was submitted as input under circular plasmid. The region of insertion of insert fragment is depicted in Fig. 4.2. Specific restriction enzymes were then selected from the custom digest displayed on the web server. The reaction mixture (10 µL) for restriction digestion consisted of 1 X restriction buffer (Thermo Scientific, USA), 5 units of each restriction enzyme (Thermo Scientific, USA) and approximately 200 ng/µL of recombined plasmid. Restriction digestion was carried out at 37°C for 1 h. The restriction digests were visualised on 1.2% agarose gel containing 0.5 µg.mL^{-1} of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The restriction profile of the recombined plasmid is given in Table. 4.6.

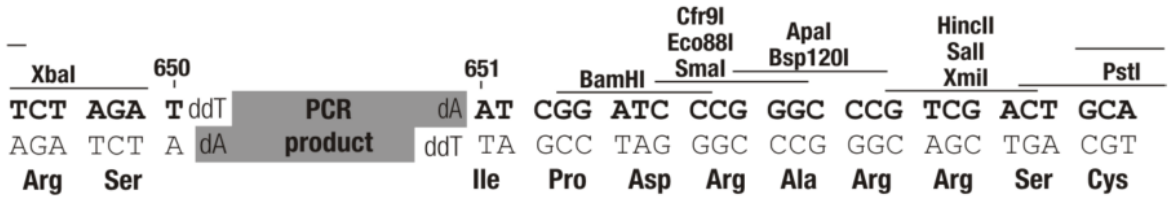


Fig.4.2. Region of insertion of internal fragment of the gene with ddA overhang in the pTZ57R/T vector sequence.

Table 4.6. Restriction profile of the recombined vectors used for mutagenesis in *R. solanacearum*.

Recombined plasmid	Restriction enzyme	Band size (bp)
	T3SS	
hrpBpTZ57R/T	*Rsa* I	2333, 1066, 72
	Bam HI/*Eco* RI double digest	2847, 403, 221
	Sac I/*Not* I double digest	3361, 110
	Eco RI	3250, 221
hrcVpTZ57R/T	*Bam* HI/*Eco* RI double digest	2847, 802
	Rsa I	1987, 1066 483, 113
	T6SS	
TssMpTZ57R/T	*Bam* HI	3569
	Pst I	2951, 618
	Eco RI	3078, 498
	Xba I/*Hind* III double digest	2840, 729
TssHpTZ57R/T	*Apa* I	3850
	Bam HI/*Eco* RI double digest	2847, 733
	Pst I	3026, 554
HcppTZ57R/T	*Apa* I	3307
	Bam HI/*Eco* RI double digest	2847, 460
	Pst I/ *Sac* II double digest	2975, 332
VgrG3pTZ57R/T	*Apa* I	3605
	Bam HI/*Eco* RI double digest	2847, 758
	Rsa I	2539, 1066
OmpApTZ57R/T	*Apa* I	3452
	Bam HI/*Eco* RI double digest	2847, 605
	Sac II	3221, 231
	Sac I	3229, 223

4.2.4. Mutagenesis of *R. solanacearum*

4.2.4.1. Preparation of electrocompetent cells

A single colony of *R. solanacearum* isolate Rs-09-161 was inoculated in BG medium and grown at RT overnight under constant shaking at 160 rpm. This culture was inoculated to a fresh BG broth (100 mL) to an initial OD_{600nm} of 0.15 and allowed to grow under shaker conditions. Actively growing cells were harvested at OD_{600nm}= 0.5 and electrocompetent cells were prepared under ice cold conditions. Culture broth was centrifuged at 8000 rpm for 10 min at 4°C. The culture supernatant was discarded and the cells were re-suspended in equal volume of cold MilliQ water. The centrifugation steps were repeated successively by re-suspending the cells in half volume of MilliQ water, one fourth volume of 10% glycerol and finally in $1/100^{th}$ volume of 10% glycerol solution respectively.

4.2.4.2. Electroporation and selection of mutants

The 0.1 cm electroporation cuvettes were used for electroporation. The cuvettes were pre-chilled on ice. Electrocompetent cells (100 µL) were mixed with 2 µg of plasmid and electroporated at 2 kV in micropulser (Bio-Rad). To the cuvettes 700 µL of BG broth was added immediately after electroporation and the contents were transferred to a fresh tube. The tubes were maintained on shaker conditions at 200 rpm for 2 h at RT. The cells were pelleted at 4000 rpm for 5 min and resuspended in 100 µL of BG broth. The suspended cells were plated on BG agar plates containing ampicillin (100 $\mu g.mL^{-1}$) and incubated for 48-72 h. The colonies obtained were subcultured on fresh BG agar ampicillin plates. Mutants were routinely cultured on BG-ampicillin (100 $\mu g.mL^{-1}$) agar plates.

4.2.4.3. Confirmation of *R. solanacearum* mutants

Positive colonies obtained in the presence of antibiotic were confirmed for integration of the plasmid and for the orientation of insert at the genomic level using specifically

designed diagnostic primer and M13 primers for mutants. The extraction of genomic DNA was carried out using procedure as described by Ramesh *et al.* (2014a). The detailed procedure for genomic DNA isolation is given in section 4.2.2.2. The sequences of the primers and standardised PCR conditions are given in table 4.7 and 4.8 respectively. All the amplifications were performed in Mastercycler Pro (Eppendorf, GmBH). The amplicons were visualised on 1.0% agarose gel containing 0.5 $\mu g.mL^{-1}$ of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The confirmed mutants were then maintained as 30% glycerol stock at -80°C. The diagrammatic representation of insertional mutagenesis by homologous recombination is given in Fig. 4.3.

Table 4.7. List of diagnostic primers used in the study

Gene	Primer	Primer sequence (5'-3')	Amplicon (bp)
		T3SS	
hrpB	HrpB-D	CACCAACGACCAGATGC	1628
	M13F	GTAAAACGACGGCCAGT	
hrcV	HrcV-D	GGACGAAGTCAAGCGCGAGC	1624
	M13R	CAGGAAACAGCTATGAC	
		T6SS	
tssM	TssM-D	GACCCCCGATTTCCGATTCGT	1101
	M13F	GTAAAACGACGGCCAGT	
tssH	TssH-D	GTCAGGACGTGGCCGGCTGC	1203
	M13R	CAGGAAACAGCTATGAC	
hcp	Hcp-D	CTGCAAGAACTCGGACTACG	1014
	M13F	GTAAAACGACGGCCAGT	
vgrG3	Vgr3-D	CGGAAACGGCCAAGCTTGTC	1302
	M13F	GTAAAACGACGGCCAGT	
ompA	OmpA-D	GCCGATCCGCTCTACCGCAGC	980
	M13F	GTAAAACGACGGCCAGT	

Table 4.8. Details about the reaction mixture and PCR conditions for confirmation of mutagenesis of gene in *R. solanacearum*.

Gene	Reaction mixture	PCR conditions
	T3SS	
hrpB	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 20 µg BSA, 0.5 U Taq DNA polymerase and 50 ng µL of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 1 min 30 s and final extension of 10min at 72°C
hrcV	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1X assay buffer, 3% DMSO, 20 µg BSA, 0.5 U Taq DNA polymerase and 50 ng of the DNA template.	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 1 min 30 s and final extension of 10min at 72°C.
	T6SS	
tssH	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 5% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30s , 72°C for 1 min 15 s and final extension of 10min at 72°C.
hcp	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min 15 s and final extension of 10 min at 72°C
vgrG3	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 1 min 15 s and final extension of 10min at 72°C.

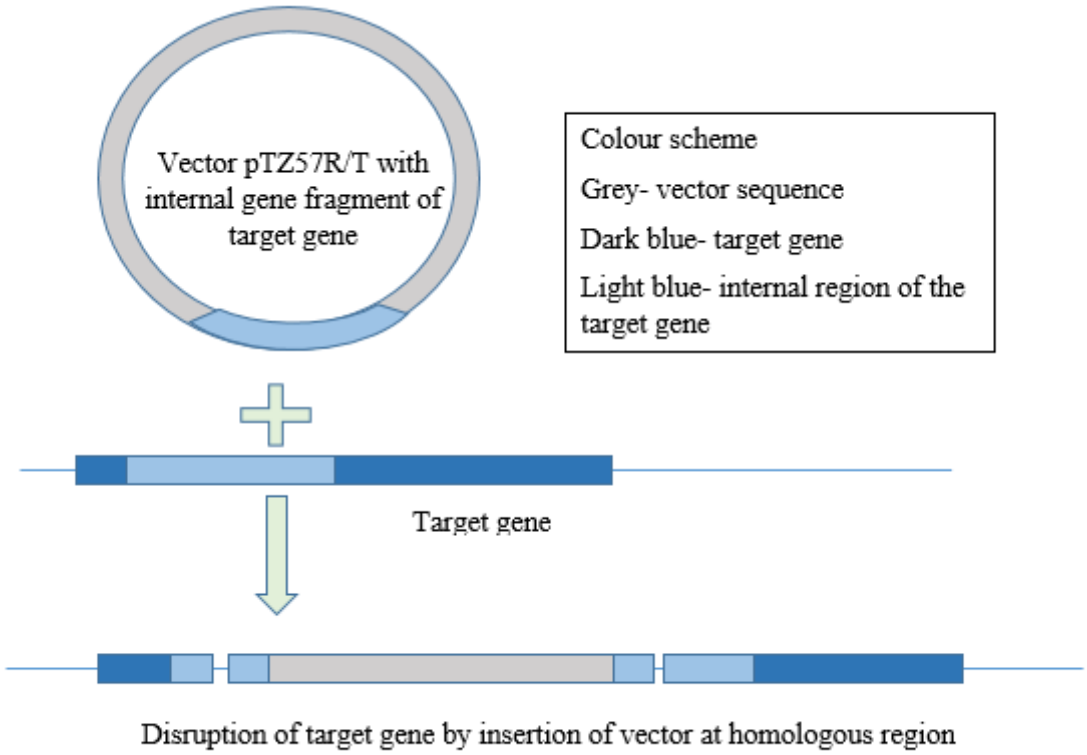


Fig. 4.3. Diagrammatic representation of mutagenesis of gene by homologous recombination.

4.2.5. Stability studies of the Rs-hrpB$^-$ and Rs-hrcV$^-$ mutant

A single colony of Rs-hrpB$^-$ and Rs-hrcV$^-$ was inoculated in 10 mL of BG broth and maintained under shaker conditions at room temperature without ampicillin for 24 h. After 24 h, 1 mL of the culture aliquot was diluted to O.D.$_{600nm}$= 0.1 and 10^{-6} dilution was plated on BG agar with and without ampicillin and incubated for 48 h. Two replications were maintained for each dilution plated on each medium. The numbers of colonies obtained on the two mediums were counted. Every day for 10 consecutive days 100 µL of culture was sub-cultured into fresh BG broth without antibiotic and the stability of the plasmid in the absence of antibiotic was recorded by plating the culture.

4.3. Results and Discussion

4.3.1. Selection of isolate

R. solanacearum wild type isolate Rs-09-161 was selected as the parent isolate in this study. It is a highly virulent isolate, isolated from the west coast of India and the genome sequence of this isolate is available (Ramesh *et al.*, 2014b). Rs-09-161 forms white fluidal irregular colonies and exhibit a pink whirling pattern at the centre on TZC medium (Fig. 4.4). Details of the plasmid and stains used in this study are given in table 4.9.

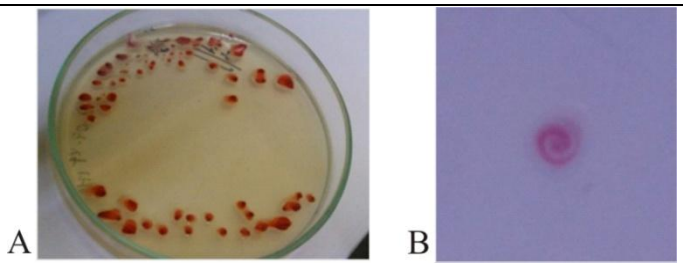


Fig.4.4. (A) Typical *R. solanacearum* colonies on BG media supplemented with TZC. (B) Whirling pattern exhibited by *R. solanacearum* colonies

Table 4.9. List of plasmids and isolates used in the study

Isolates	Relevant genotypes
E.coli JM107	
Ec-hrpB	hrpB::pTZ57R/T
Ec-hrcV	hrcV::pTZ57R/T
Ec-TssM	tssM::pTZ57R/T
Ec-TssH	tssH::pTZ57R/T
Ec-Hcp	hcp::pTZ57R/T
Ec-VgrG3	vgrG3::pTZ57R/T
Ec-OmpA	ompA::pTZ57R/T
R. solanacearum	
Rs-09-161	WT
Rs-hrpB^{-}	hrpB::pTZ57R/T mutant
Rs-hrcV^{-}	hrcV::pTZ57R/T mutant
Rs-TssM^{-}	tssM::pTZ57R/T mutant
Rs-TssH^{-}	tssH::pTZ57R/T mutant
Rs-Hcp^{-}	hcp::pTZ57R/T mutant
Rs-VgrG3^{-}	vgrG3::pTZ57R/T mutant
Rs-OmpA^{-}	ompA::pTZ57R/T mutant
Plasmid	
pTZ57R/T	Fermentas TA cloning vector
hrpBpTZ57R/T	*hrpB* gene fragment cloned in the TA vector
hrcVpTZ57R/T	*hrcV* gene fragment cloned in the TA vector
TssMpTZ57R/T	*tssM* gene fragment cloned in the TA vector
TssHpTZ57R/T	*tssH* gene fragment cloned in the TA vector
HcppTZ57R/T	*hcp* gene fragment cloned in the TA vector
VgrG3pTZ57R/T	*vgrG3* gene fragment cloned in the TA vector
OmpApTZ57R/T	*ompA* gene fragment cloned in the TA vector

4.3.2. PCR amplification of the internal fragments

Seven genes were selected for the mutagenesis in *R. solanacearum*isolate Rs-09-161. These included *hrpB, hrcV, tssM, tssH, hcp, vgrG3 and ompA*. The genes *hrpB* and h*rcV* belong to the T3SS. The genes *tssM, tssH, hcp, vgrG3 and ompA* belong to the T6SS. The DNA was extracted from Rs-09-161 and quantified. The internal fragments of the selected genes were amplified and purified. The amplification of internal fragment is shown in Fig. 4.5.

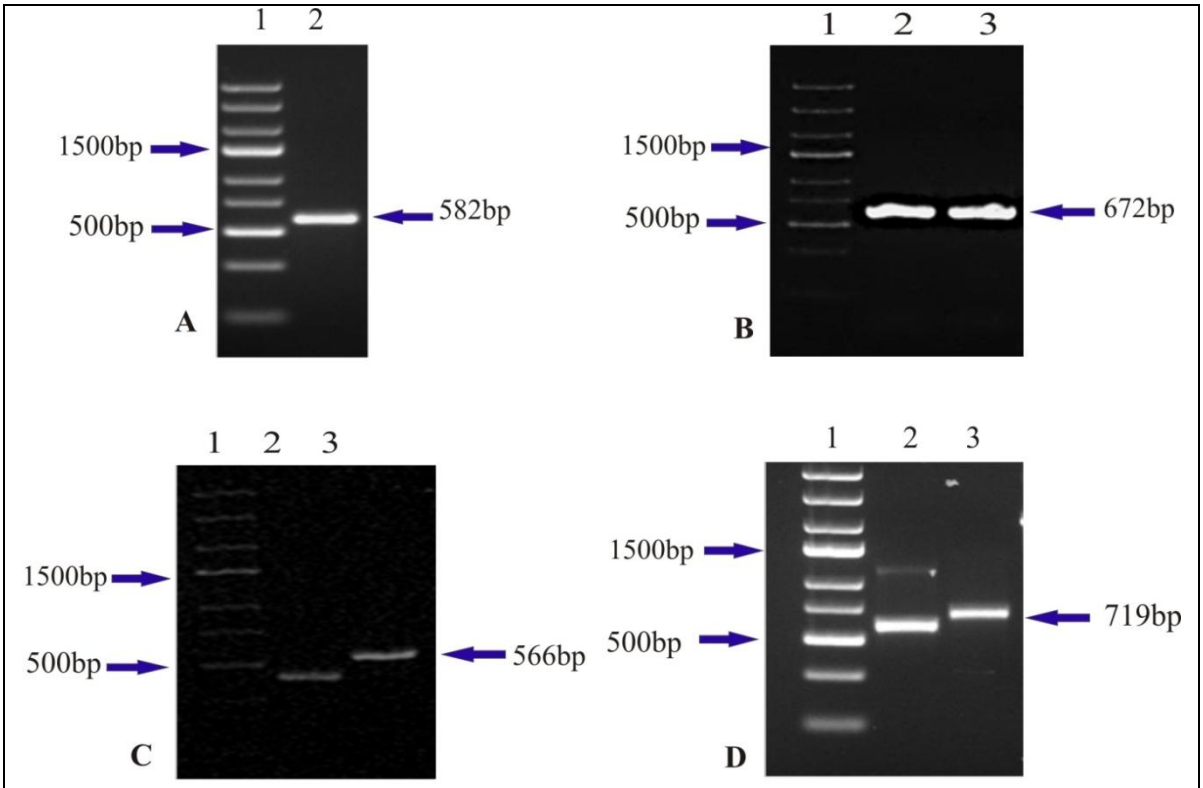


Fig. 4.5.Amplification of internal fragments of the genes selected for mutagenesis. (A) Amplification of internal fragment of *hrpB* gene. Lane 1: Ladder, lane 2: *hrpB* internal fragment (582 bp). (B) Amplification of internal fragment of *tssM* gene. Lane 1: Ladder, lane 2-4: 672 bp. (**C**) Amplification of internal fragment of *hcp* and *ompA* gene. Lane 1: Ladder, lane 2: Amplification of internal fragment of *hcp* gene (421 bp), lane 3: Amplification of internal fragment of *ompA* gene (566 bp). (D) Amplification of internal fragment of *tssH* and *vgrG3* gene. Lane 1: Ladder, lane 2: *tssH* internal fragment (694 bp), lane 3: *vgrG3* internal fragment (719 bp).

***Ladder:** 100 bp-5 Kb, GeneRuler Express DNA Ladder

4.3.3. Development of clones with pTZ57R/T vector in *E.coli*.

The amplified internal fragments were cloned in the linear vector pTZ57R/T. White colonies were selected as positive clones after transformation by blue and white selection. The positive control exhibited all white colonies whereas the negative control exhibited all blue colonies.

The non-template dependant terminal transferase activity of the thermostable Taq DNA polymerase adds adenosine overhang at both the 3'-end of the fragments amplified during PCR. These fragments get cloned in the linearized vector pTZ57R/T very efficiently with the help of 3'-ddT overhangs present on its either sides (Zhou and Gomez-Sanchez, 2000). The overhangs present on the linearized vector also prevent the recircularization of the vector and thus does not yield any colonies without the insert fragment. TA cloning vectors are widely used in cloning (Minamito *et al.*, 2012; Liu *et al.*, 2005)

The presence of internal gene fragment in the colonies was confirmed by colony PCR of Ec-hrpB, Ec-hrcV, Ec-TssM, Ec-TssH, Ec-Hcp, Ec-VgrG3 and Ec-OmpA which gave the characteristic amplification bands at 582 bp, 763 bp, 672 bp, 694 bp, 421 bp, 719 bp and 566 bp respectively (Fig. 4.6.). The colonies were cultured in LB broth with ampicillin and the recombined plasmid was isolated. The concentration of the isolated plasmid was in the range of 150-300 ng/µL. Restriction digestion of the isolated plasmid confirmed the orientation of the insert. The restriction profile of the recombined plasmids is depicted in Fig.4.7.

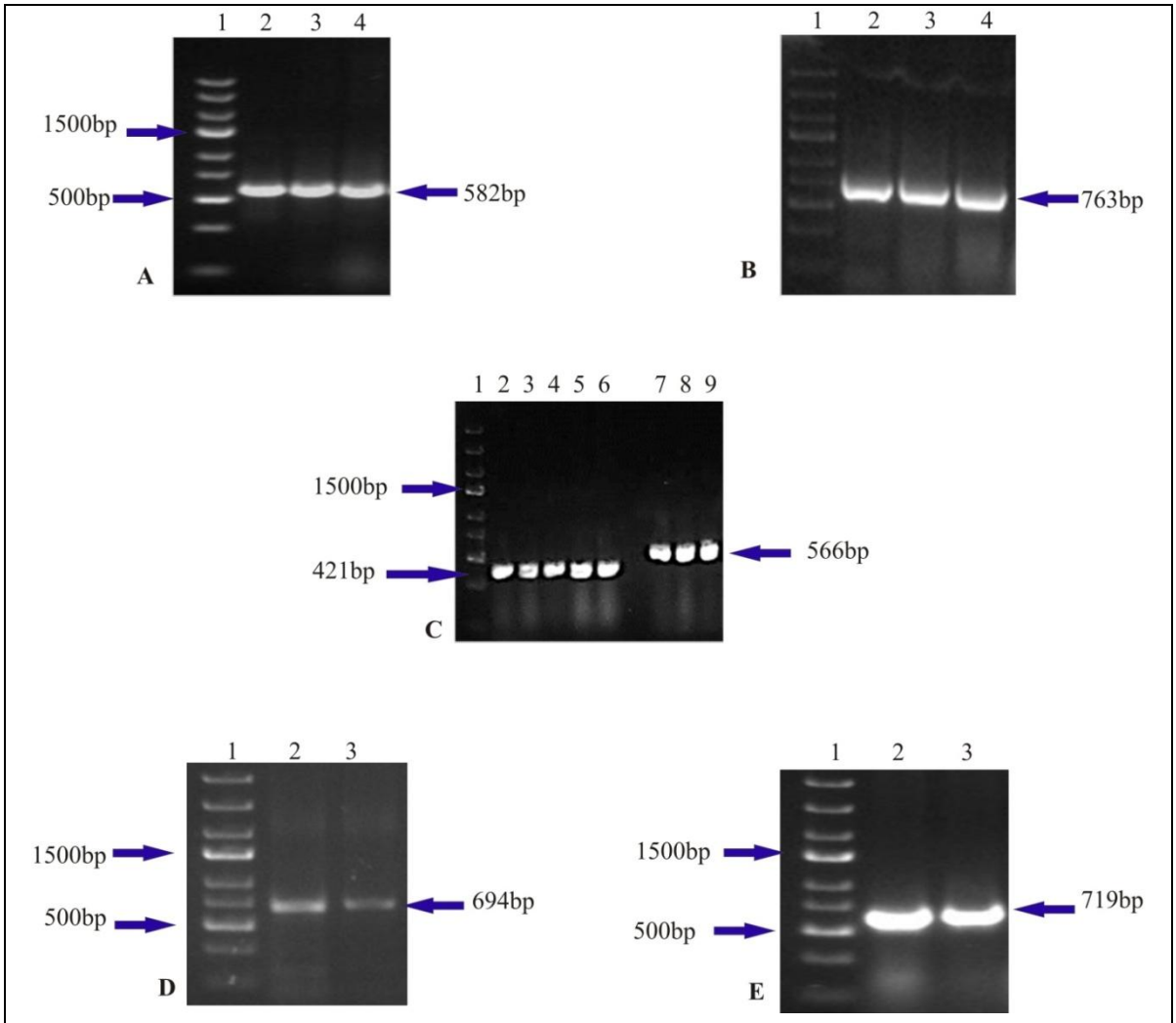


Fig. 4.6. Colony PCR for the confirmation of the selected T3SS and T6SS gene insert in *E.coli* colonies. (A) Confirmation of *hrpB* insert in Ec-HrpB colonies. Lane 1: Ladder, Lane 2-4 582 bp. (B) Confirmation of *hrcV* insert in Ec-HrcV colonies. Lane 1: Ladder, Lane 2-4: 763 bp. (C) Confirmation of and *hcp* and *ompA* gene fragment Ec-OmpA and Ec-Hcp colonies. Lane 1: Ladder, Lane 2-6: *hcp* fragment (421 bp), Lane 7-9: *ompA* fragment (566bp). (D) Confirmation of *tssH* gene fragment in Ec-TssH colonies. Lane 1: Ladder, Lane 2-4: 694 bp. (E) Confirmation of *vgrG3*gene fragment in Ec-VgrG3 colonies, Lane 1: Ladder, Lane 2-3: 719 bp.

***Ladder:** 100 bp-5 Kb, GeneRuler Express DNA Ladder

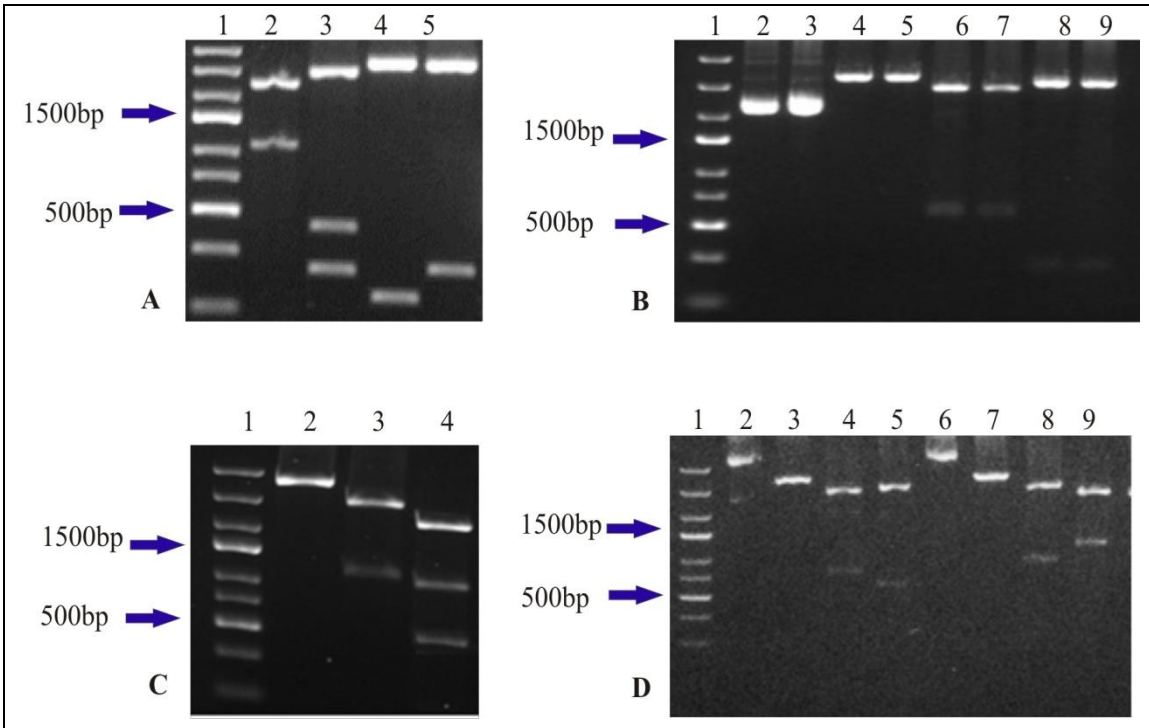


Fig.4.7.Restriction digestion of the recombined plasmids containing the internal fragment of selected genes. (A)Restriction digestion of plasmid hrpBpTZ57R/T which contains the internal fragment of *hrpB* gene cloned into it. Lane 1: ladder; lane 2: *Rsa* I digest with 3 bands at 2333 bp, 1066 bp and 72 bp; lane 3: *Bam* HI/*Eco* RI double digest with 3 bands of 2847 bp, 403 bp and 221 bp; lane 4: *Sac* I/*Not* I double digest with 2 bands of 3361 bp and 110 bp; lane 5: *Eco* RI digest with 2 bands at 3250 bp and 221 bp. (B)Restriction digestion of plasmid ompApTZ57R/T which contains the internal fragment of *ompA* gene cloned into it. Lane 1: ladder; lane 2, 3: uncut plasmid ompApTZ57R/T; lane 4, 5: *Apa* I linearization with 3452 bp; lane 6, 7: *Bam* HI/*Eco* RI double digest with 2 bands of 2847 bp, 605 bp; lane 8, 9: *Sac* I digest with 2 bands at 3229 bp and 223 bp. (C) Restriction digestion of plasmid hrcVpTZ57R/T which contains the internal fragment of *hrcV* gene cloned into it. Lane 1: Ladder; lane 2: uncut plasmid hrcVpTZ57R/T; lane 3: *Bam* HI/*Eco* RI double digest with 2 bands of 2847 bp, 802 bp; lane 4: *Rsa* I digest with 4 bands at 1987 bp, 1066 bp 483 bp and 113 bp. (D) Restriction digestion of plasmid TssHpTZ57R/T and VgrG3pTZ57R/T which contains the internal fragment of *tssH* and *vgrG3* gene cloned into it respectively. Lane 1: ladder; lane 2: uncut TssHpTZ57R/T; lane 3: *Apa* I linearization with 3850 bp; lane 4: *Bam* HI/*Eco* RI double digest with 2 bands of 2847 bp and 733bp; lane 5: *Pst* I digest with two bands at 3026 bp and 554 bp; lane 6: uncut VgrG3pTZ57R/T; lane 7: *Apa* I linearization with 3605 bp; lane 8: *Bam* HI/*Eco* RI double digest with 2 bands of 2847 bp and 758 bp; lane 9: *Rsa* I digest with two bands at 2539 bp and 1066 bp.

***Ladder:** 100 bp-5 Kb, GeneRuler Express DNA Ladder

4.3.4. Development of *R. solanacearum* mutants

The recombined plasmids from Ec-hrpB, Ec-hrcV, Ec-TssM, Ec-TssH, Ec-Hcp, Ec-VgrG3 and Ec-OmpA were used as the vectors for mutagenesis. Electroporation of electrocompetent Rs-09-161 cells with individual recombined plasmid generated the mutant of the specific gene. The vector pTZ57R/T lacks the oriC for *R. solanacearum* and hence cannot replicate in Rs-09-161. When the recombined vector containing an internal fragment is transferred into the *R. solanacearum* cell, due to the sequence similarity shared by the cloned insert fragment and the target gene, the plasmid integrates itself into the target gene by homologous recombination. The integration of the plasmid within the target gene lead to its disruption by insertional mutagenesis. The mutant colonies appeared on BG agar plates with ampicillin (100 $\mu g.mL^{-1}$) 48-72 h after electroporation. This is a simple, time saving and cost efficient method and can be easily used for the development of insertional mutants for a large number of genes. The colonies were subcultured and DNA was extracted from the colonies for the confirmation of mutagenesis.

The insert fragment is flanked by M13 primers nucleotide sequence on the either side. The diagnostic primer designed 1000 bp upstream of the gene located in the genome pairs with either of the M13 primers based on the orientation of the plasmid and amplifies the region directing the disruption of the gene at the desired site. In case of T3SS mutants, Rs-hprB$^-$ was confirmed by the presence of 1628 bp band with M13 forward primer and Rs-hrcV$^-$ was confirmed by 1624 bp band with M13 reverse primer. In the T6SS mutants, Rs-TssM$^-$ was confirmed by the presence of 1101 bp band with M13 forward primer, Rs-TssH$^-$ by 1203 bp with M13 reverse primer, Rs-Hcp$^-$ by 1014 bp with M13 forward primer and Rs-OmpA$^-$ by 980 bp with M13 forward primer respectively. The confirmation of insertional mutagenesis in *R. solanacearum*is depicted in fig. 4.8.

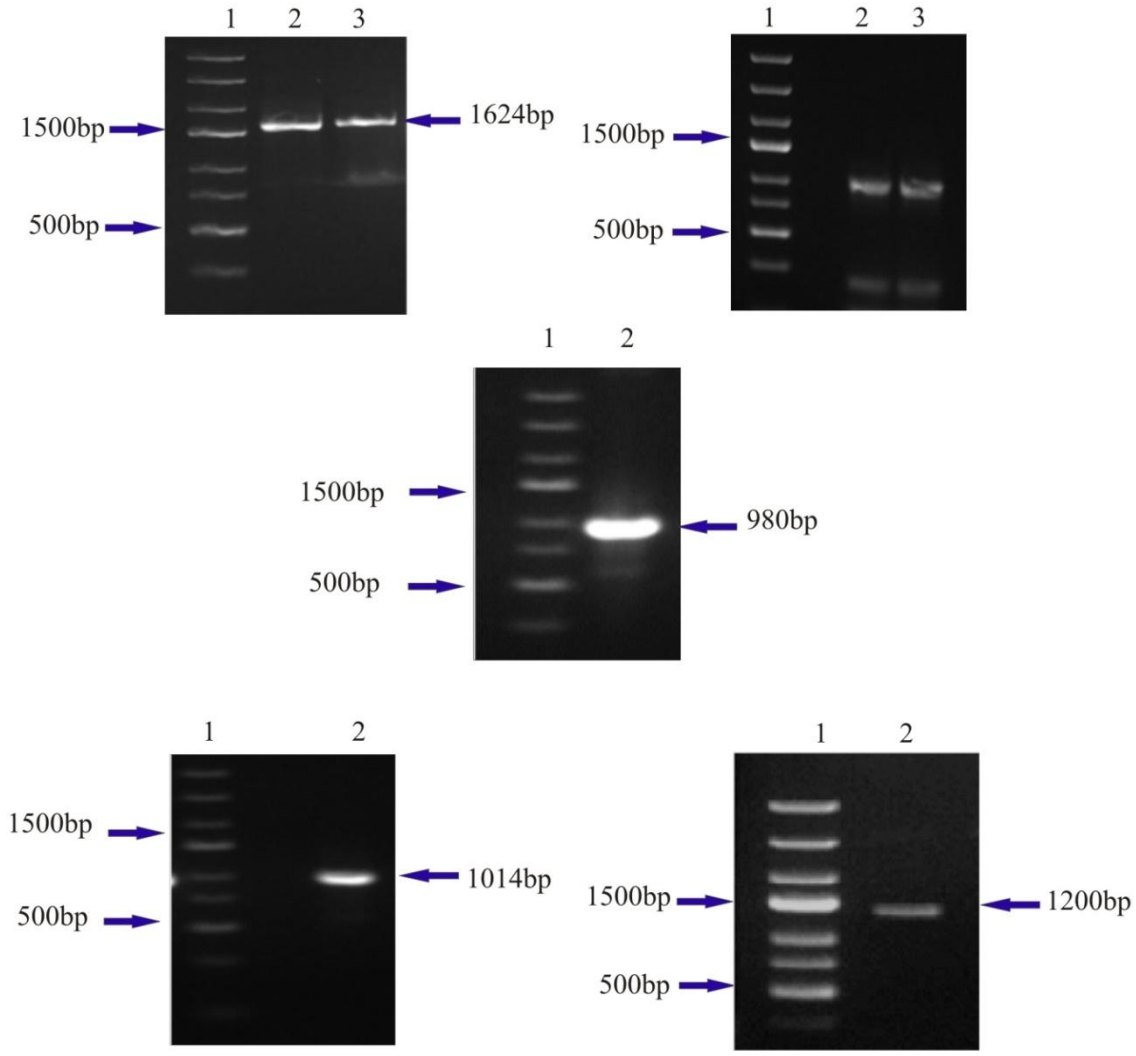


Fig. 4.8.Confirmation of insertional mutagenesis in *R. solanacearum* Rs-09-161. (A) Lane 1: Ladder, lane 2: 1628 bp obtained by disruption of *hrpB* gene in Rs-hrpB^{-}; lane 3: 1624 bp obtained by disruption of *hrcV* gene in Rs-hrcV^{-}. (B) Lane 1: ladder; lane 2-3: 1101 bp obtained by disruption of *tssM* gene in Rs-TssM^{-}. (C) Lane 1: ladder; lane 2: 980 bp obtained by disruption of *ompA* gene in Rs-OmpA^{-}. (D) Lane 1: ladder; lane 2: 1014 bp obtained by disruption of *hcp* gene in Rs-Hcp^{-}. (E) Lane 1: ladder; lane 2: 1203 bp obtained by disruption of *tssH* gene in Rs-TssH^{-}.

4.3.5. Stability of mutants

The stability of the integrated plasmid was studied with Rs-hrpB$^-$ and Rs-hrcV$^-$. Both, Rs-hrpB$^-$ and Rs-hrcV$^-$, were found to be stable in the absence of antibiotic for a period of 10 days. There was no significant difference in the population of mutants on BG medium with antibiotic and without antibiotic on each day. Population of Rs-hrpB$^-$ was log 10.72 CFU.mL^{-1} on BG medium without ampicillin and log 10.15 CFU.mL^{-1} on BG medium with ampicillin after 10 days. The population of Rs-hrcV$^-$ was log 9.9 CFU.mL^{-1} on BG medium without ampicillin and log 9.5 CFU.mL^{-1} on BG with ampicillin after 10 days (Table 4.10). This indicates the plasmid was stably integrated into the genome and would not lose in the plant where the antibiotic selection is absent.

Table 4.10. Population of *R. solanacearum* mutant Rs-hrpB$^-$ and Rs-hrcV$^-$ in the absence of antibiotic selection to study the stability

Days	Rs-hrpB$^-$(log CFU.mL^{-1})		Rs-hrcV$^-$(log CFU.mL^{-1})	
	BG	BG withAmpicillin	BG	BG with Ampicillin
1	10.51	10.04	10.58	10.63
2	10.15	9.79	10.48	10.52
3	10.31	9.78	10.45	10.41
4	10.52	10.47	10.50	10.18
5	10.63	10.10	10.43	10.25
6	10.43	9.92	10.34	10.07
7	10.82	10.45	9.80	9.65
8	10.76	10.10	9.70	9.80
9	10.95	10.39	10.01	9.71
10	10.72	10.15	9.90	9.54

Mutants of *R. solanacearum*have been developed for many genes like *pehA*, *pehB*, *egl*, *pme* and *eps* (Huang and Allen, 2000; Saile, 1997). *hrpB* is the transcriptional regulator which controls the expression of T3SS and the T3Es and *hrcV* is a transmembrane protein that forms a component of hrp Pili, the needle like apparatus which is involved in translocation of the T3Es into the host cells. Mutant of the *hrpB* gene produced phenotype same as wild type. Mutant of *hrcV* gene produced less fluidity as compared to wild type (Fig. 4.9).

Fig. 4.9. Phenotype of wild type Rs-09-161 and T3SS mutants: Rs-hrpB^{-} and Rs-hrcV^{-}.

Mutants of *hrpB* and *hrcV* genes were produced by insertion of Tn5-B20 and Ω-Cm interposon (*Genin et al., 1992; Etchebar et al., 1998*). The mutant of *hrpB* gene is poorly infective, weakly invasive, and display limited multiplication in the intercellular spaces of the root cortex and vascular system of tomato plants (*Vasse et al., 2000*). Mutants of the T3SS are considered to be nonpathogenic. The *hrpB* and *hrcV* mutants are used as the T3SS deficient counterparts in validation of T3Es. The mutants developed in this study will help in validating the translocation of putative T3E gene. In *R. solanacearum*, work on T6SS is limited to the gene *tssM* and *tssB* (Zhang *et al.*, 2012; 2014). In this study, mutants of *tssM, tssH, hcp, vgrG3* and *ompA* were developed. These mutants can be used to analyze the importance of T6SS in virulence of *R. solanacearum.*

4.3.6. Conclusion

R. solanacearum mutants of the T3SS and T6SS were developed through insertional mutagenesis by homologous recombination and confirmed with the help of diagnostic primer. In the T3SS, mutants of *hrpB* and *hrcV* genes were developed. These mutants can be used as T3SS deficient mutant (Rs-hrpB$^-$) and the secretion deficient mutant (Rs-hrcV$^-$) in the validation of T3E. These mutants can also be used for virulence determination on susceptible hosts. In the T6SS, mutants of *tssM, tssH, hcp, ompA* and *vgrG3* genes were developed. These mutants can be used to study the role of T6SS genes in the virulence of *R. solanacearum* with the help of plant assays.

CHAPTER V

Virulence determination of mutants through functional assays or plant assays

5.1. Introduction

A large number of plant diseases are caused by bacterial pathogens. Some of these include bacterial wilt (*R. solanacearum*), crown gall (*A. tumifaciens*), bacterial leaf blight, black rot (*Xanthomonas spp.,*), fire blight (*E. amylovora*), etc (Mansfield *et al.,* 2012). Pathogens possess within them certain factors which makes it virulent on its susceptible hosts. Hence it is important to identify various virulence factors present in a pathogen which promote its growth and disease causing ability.

Bacterial wilt by *R. solanacearum* is reported globally to cause severe economic losses (Assefa *et al.,* 2015). Extensive research conducted on this bacterium has shed light on its virulence mechanisms. Among the major virulence factors of *R. solanacearum* are the EPS, PCWDE, chemotaxis, swimming and twitching motility (Genin and Denny, 2012). Also present within the organism is the T3SS, which acts as its pathogenicity determinant (Hueck, 1998). The role played by these factors in the virulence of bacterium is proved with the help of mutants. For instance, the EPS deficient mutants are avirulent and poorly infective (Araud-Razou *et al.,* 1998). EPS provides the bacterium with an extracellular fluidal layer which impairs the transport of water in the vascular tissue (Genin and Denny, 2012). The polygalacturonases play an important role in host colonisation and virulence. The mutants of genes *pehA* and *pehB* display significantly less population and reduced virulence on tomato plants (Huang and Allen, 2000). However, a *pehA/pehB/pehC* triple mutant of polygalacturonases is more virulent, because, *pehC* is involved in degrading the plant cell wall triggering the host defence through oligogalacturonides (Liu *et al.,* 2005).

The T3SS is the pathogenicity determinant of *R. solanacearum* and is encoded by the *hrp* regulon. It injects a large number of T3E proteins into the host cells which makes the host susceptible or generates a hypersensitive response in resistant plants (Coll and Valls,

2013). The mutants of the T3SS are reported to be non–pathogenic and are used in the validation of putative T3Es (Cunnac *et al.,* 2004b; Mukaihara *et al*., 2010; Monteiro *et al.,* 2012a).

The T6SS is recently identified among gram negative bacteria and is thought to be involved in pathogenesis, bacterial interactions and competition (Silverman *et al.,* 2012). In *R. solanacearum*, the T6SS genes have been proved to be involved in virulence, but there is limited data available on the contribution of T6SS genes in virulence of *R. solanacearum*. Mutants of *tssM* and *tssB* genes of T6SS have led to the attenuation of virulence on tomato plants in comparison to the wild type strain (Zhang *et al.,* 2012; 2014).

Since *R. solanacearum* is a pathogen, any defect in the genes involved in contributing towards the virulence will lead to reduction in its ability to cause disease. This reduction will have a direct effect on its virulence and can be checked through plant assays. Development of insertional mutants of the T3SS and the T6SS was discussed in the previous chapter. The mutants of T3SS were developed with the intention of validating a putative T3E. The mutants of the T6SS were developed to study the role of T6SS in the virulence of *R. solanacearum*. Keeping these things in mind the objective-"Virulence determination of mutants through functional assays or plant assays"was proposed.

5.2. Materials and methods

5.2.1. *R. solanacearum* mutants

The following seven insertional mutants of *R. solanacearum* (Table 5.1) developed in chapter IV were used for assaying their virulence through plant assays. These mutants are developed from the wild type strain of *R. solanacearum* Rs-09-161. The genes *hrpB* and

hrcV belong to the T3SS of *R. solanacearum*. These mutants were developed to use it as base material in the validation of T3E protein (Chapter VI). The genes *tssM, tssH, hcp, vgrG3* and *ompA* genes belong to the T6SS system of *R. solanacearum*.

Table 5.1.*R. solanacearum* mutants used in the virulence assay.

R. solanacearum mutant	Genotype
	T3SS
Rs-hrpB$^-$	hrpB::pTZ57R/T mutant
Rs-hrcV$^-$	hrcV::pTZ57R/T mutant
	T6SS
Rs-TssM$^-$	TssM::pTZ57R/T mutant
Rs-TssH$^-$	TssH::pTZ57R/T mutant
Rs-Hcp$^-$	Hcp::pTZ57R/T mutant
Rs-VgrG3$^-$	VgrG3::pTZ57R/T mutant
Rs-OmpA$^-$	OmpA::pTZ57R/T mutant

5.2.2. Plant material

The virulence of mutants was assayed on brinjal and tomato. Highly susceptible brinjal cultivar (*Agassaim*), tomato varieties (*Pusa Ruby*, *Arka Vikash*) and moderately resistant tomato variety (*Arka Rakshak*) were used in the virulence assays. The Seedlings were raised in the greenhouse maintained at 30°C during the day with 60% relative humidity, 16 h of light and 8 h of dark period. The seeds were sown in nursery trays containing potting mixture of red soil, FYM and sand in the ratio of 2:1:1 with regular watering. After twenty days, the seedlings were transferred to bigger pots containing the potting mixture as mentioned above. Thirty five-days old seedlings were used for *R. solanacearum* inoculation.

5.2.3. Virulence determination of *R. solanacearum* mutants

The virulence of the *R. solanacearum* mutants was assayed by soil drench inoculation and petiole inoculation. The experiments were conducted separately for T3SS mutants and T6SS mutants. Wild type strain Rs-09-161 was used as a positive control and sterile water was used as a negative control in T3SS mutant evaluation assays while Rs-hrcV$^-$ was used as a negative control (non-pathogenic strain) in T6SS mutant evaluation assays. Two different methods of inoculation were used in plant assays: soil drench inoculation and petiole inoculation. Each experiment was repeated at least twice with minimum two replications and each replication had five plants each. The seedlings were observed for wilt symptoms for 21 days post inoculation (DPI). The wilt incidence was recorded and expressed as percentage wilt.

5.2.3.1. T3SS mutants of *R. solanacearum*

The T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$, developed in the Chapter IV were assayed for its virulence on brinjal and tomato seedlings. The highly susceptible cultivar of brinjal (*Agassaim*) and variety of tomato (*Pusa Ruby*) were used for plant assays.

5.2.3.1.1. Soil drench inoculation

A single colony of *R. solanacearum* wild type Rs-09-161 and its T3SS mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$ was grown in 100 mL of BG broth (Appendix II) at 150rpm for 18-20 h at RT. The culture was centrifuged at 8000 rpm, and the pellet was re-dissolved in 100 mL of PBS (Appendix III). The optical density of the culture was measured at $O.D._{600nm}$ and the cultures were diluted to 10^8 CFU.mL^{-1} (O.D.600nm=0.1). Ten millilitre of this inoculum was drenched around the root zone (Ramesh *et al.*, 2016).

The experiment was conducted twice in brinjal cultivar (*Agassaim*). Experiment 1 was conducted with two replications and experiment 2 was conducted with three replications

respectively. Each replication had five plants. In case of tomato (*Pusa Ruby*), the experiment was conducted twice with three replications each.

5.2.3.1.2. Petiole inoculation

In petiole inoculation, the seedlings were inoculated on the third leaf petiole from the shoot apex. The petiole of the seedlings was cut 2 cms away from the stem, 2000 cells of *R. solanacearum* were inoculated on the petiole of tomato and 500 cells on brinjal (Asolkar and Ramesh, 2018b). The experiment was conducted twice with brinjal cultivar (*Agassaim*), with two and three replications. For virulence determination in tomato (var. *Pusa Ruby*), the experiment was conducted three times with two replications in experiment 1 and 3 replications in experiment 2, 3. The total number of experiments performed with T3SS mutants with respect to the plant hosts and method of inoculation are given in table 5.2.

Table 5.2. Number of experiments in virulence assays of T3SS mutants.

Crop	Variety	Varietal Reaction	Method of Inoculation	No. of experiments
Brinjal	*Agassaim*	Susceptible	Soil drench	2
Tomato	*Pusa Ruby*	Susceptible	Soil drench	2
Brinjal	*Agassaim*	Susceptible	Petiole	2
Tomato	*Pusa Ruby*	Susceptible	Petiole	3

5.2.3.1.3. Colonization of *R. solanacearum* T3SS mutants in brinjal

Colonisation of *R. solanacearum* wild type strain Rs-09-161 and mutants Rs-hrpB$^-$ and Rs-hrcV$^-$ was assessed in thirty days-old highly susceptible brinjal cv. *Agassaim*. Overnight grown *R. solanacearum* strains Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$ were resuspended to 0.1 OD_{600nm} (10^8 CFU.mL^{-1}) and 10mL of this inoculum was drenched

around the root zone. Three replications were maintained for each strain. Colonisation of *R. solanacearum* was studied on day 2, 4, 6, 8 and 10 after the inoculation in experiment 1 and on day 4 after the inoculation in experiment 2. The root of the inoculated plant was cut into fine pieces of 1-2 mm and re-suspended in five mL of sterile water. The tubes were maintained under shaking at 150 rpm for 1 hour and later at 4°C for 30 min under stationary condition. Appropriate dilutions were prepared and 20µl was spot inoculated on BG agar plates containing antibiotic wherever required. The plates were incubated at RT for 14-16 h. Two replications were maintained per sample. *R. solanacearum* colonies were observed under Olympus CX41 microscope at 10 X magnification and identified by their twitching motility.

5.2.3.2. T6SS mutants of *R. solanacearum*

The highly susceptible cultivar of brinjal (*Agassaim*), variety of tomato (*Arka Vikash*) and moderately resistant variety of tomato (*Arka Rakshak*) were used in plant assays.

5.2.3.2.1. Soil drench inoculation

For soil drench inoculation, 10 mL of inoculum of 0.1 OD_{600nm} ($10^8 CFU.mL^{-1}$) was drenched around the root zone as described in 5.2.3.1.1. *Arka Vikash* and *Agassaim* seedlings were used in soil drench method. In case of *Agassaim*, two experiments were conducted with four replications in experiment 1 and three replications in experiment 2. In case of *Arka Vikash* the experiment was conducted twice with four replications.

5.2.3.2.2. Petiole inoculation

The seedlings were inoculated as described in section 5.3.2.1.2. Petiole inoculation was done in *Agassaim*, *Arka Vikash* and *Arka Rakshak*. In case of *Arka Vikash*, four experiments were conducted. Experiment 1, 3 and 4 consisted of three replications and experiment 2 consisted of four replications. In case of *Arka Rakshak* and *Agassaim*, three

experiments were conducted; experiment 1 containing four replications and experiment 2 and 3 containing three replications. The total number of experiments performed with T6SS mutants with respect to the plant hosts and method of inoculation are given in table 5.3.

Table 5.3: Number of experiments in virulence assays of T6SS mutants.

Crop	Variety	Varietal Reaction	Method of Inoculation	No. of experiments
Tomato	*Arka Vikash*	Susceptible	Petiole	4
Tomato	*Arka Rakshak*	Moderately resistant	Petiole	3
Brinjal	*Agassaim*	Susceptible	Petiole	3
Tomato	*Arka Vikash*	Susceptible	Soil drench	2
Brinjal	*Agassaim*	Susceptible	Soil drench	2

5.2.3.2.3. Statistical analysis

The data obtained in virulence assays of T6SS mutants was analysed by WASP software (http://www.ccari.res.in/wasp2.0/rbd1.php) available at ICAR-CCARI, Goa. Incidence of bacterial wilt on brinjal and tomato in each experiment was compared using CD (5%) values.

5.2.4. Inter-bacterial competition assay

In order to find out the role of T6SS mutants in bacterial killing qualitative inter-bacterial competition assay was performed. *E. coli* strain DH5α containing a circular pCR2.1 plasmid was used as a model organism in studying the inter-bacterial competition. The vector pCR2.1 (Invitrogen) has the presence of lacZα fragment in the MCS and hence in the presence of X-Gal it forms blue colonies. DH5α strain cloned with pCR2.1 vector was obtained from the culture collection of plant pathology laboratory, ICAR-CCARI. *E. coli*

was cultured on LB agar and T6SS mutants were cultured on BG agar (Appendix II). A single colony of DH5α and the T6SS mutants was inoculated in 10 mL of respective media broth and maintained under shaking condition overnight at 150 rpm. The cultures were centrifuged at 8000 rpm for 10 min. The pellet was re-dissolved in PBS and diluted to 2 x 10^4 cells.mL^{-1}. Two hundred cells of DH5α were mixed with T6SS mutants in 1:1 ratio. The isolates were then co-cultured on KB X-Gal/IPTG agar plates by spot inoculation. The plates were incubated for 48 h and observed for bacterial killing. Bacterial killing will lead to qualitative reduction of the colonies on the inoculated spot.

5.3. Results and discussion:

5.3.1. *R. solanacearum* mutants

Seven individual *R. solanacearum* mutants created by insertional mutagenesis in chapter IV were used to in this chapter. These mutants belong to the T3SS (Rs-hrpB$^-$ and Rs-hrcV$^-$) and T6SS (Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-VgrG3$^-$ and Rs-OmpA$^-$). The virulence of the mutants was assayed on brinjal and tomato seedlings.

5.3.2. Plant Material

Brinjal and tomato seedlings (*Agassaim*, *Pusa Ruby*, *Arka Vikash* and *Arka Rakshak*) were raised and maintained in the greenhouse. Twenty days-old seedlings were transferred from nursery trays to bigger pots. Five plants were transplanted per pot. Thirty days old seedlings were used for inoculation.

5.3.3. Virulence assay of *R. solanacearum* mutants on brinjal and tomato.

The virulence assays were conducted on T3SS mutants and T6SS in separate experiments.

5.3.3.1 T3SS mutants of *R. solanacearum*

The results obtained in the virulence assays of T3SS mutants are as follows:

5.3.3.1.1 Soil drench inoculation

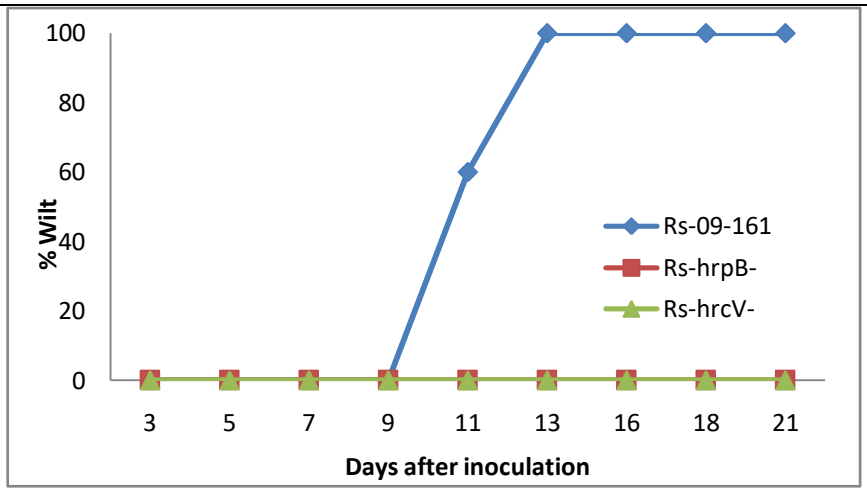
Susceptible brinjal cultivar –*Agassaim*

The virulence of *R. solanacearum* T3SS mutants, *viz.* Rs-hrpB$^-$ and Rs-hrcV$^-$ was tested by soil drench inoculation on brinjal (Cv. *Agassaim*) in two different experiments. In experiment 1, wilt incidence was observed in Rs-09-161 on 10 DAI and 100% wilt was observed on 13 DAI. In case of the mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$, no wilt incidence was observed and the mutants were non-pathogenic. In experiment 2, wilt incidence was observed on day four in the seedlings inoculated with Rs-09-161 and 100% wilt was observed on day nine. The mutant Rs-hrcV$^-$ was non-pathogenic in the second experiment but, Rs-hrpB$^-$, exhibited a wilt incidence of 6.66% on day nine. This can be considered as an exception as the wilt was observed in only one out of 15 seedlings inoculated in the second experiment. The wilt incidence caused by the T3SS mutants on brinjal in experiment 1 and 2 is represented in Fig. 5.1 (a, b). The mean values of the wilt incidence caused by the T3SS mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$ on susceptible brinjal seedlings (Cv., *Agassaim*) are represented in Fig 5.1(c). The wild type strain of *R. solanacearum*, Rs-09-161 caused 100% wilt in brinjal seedlings with drench inoculation on day 13. The mutant Rs-hrcV$^-$ was non-pathogenic and Rs-hrpB$^-$ exhibited a mean wilt percentage of 3.3%.

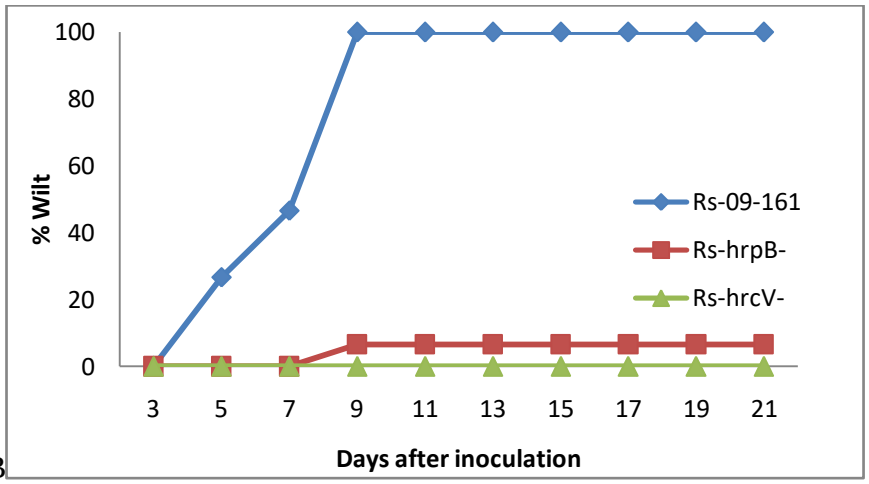
Susceptible tomato variety –*Pusa Ruby*

The wilt incidence of the mutants by soil drench inoculation on tomato (var. *Pusa Ruby*), was assayed twice with three replications in each experiments. In the individual experiments, 1 and 2, wild type strain Rs-09-161, caused wilt on day four on the highly susceptible tomato seedlings. In experiment 1, 100% wilt was incident on day seven and day 11 in experiment 2 (Fig. 5.2 a, b). This proves that Rs-09-161 is a highly virulent on susceptible tomato. The mutants, Rs-hrpB^{-} and Rs-hrcV^{-}, were non-pathogenic in both the experiments.

A



B



C

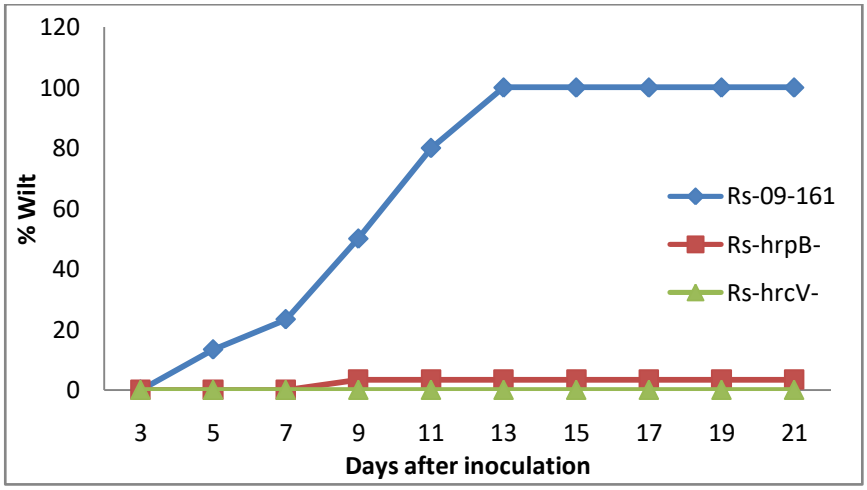
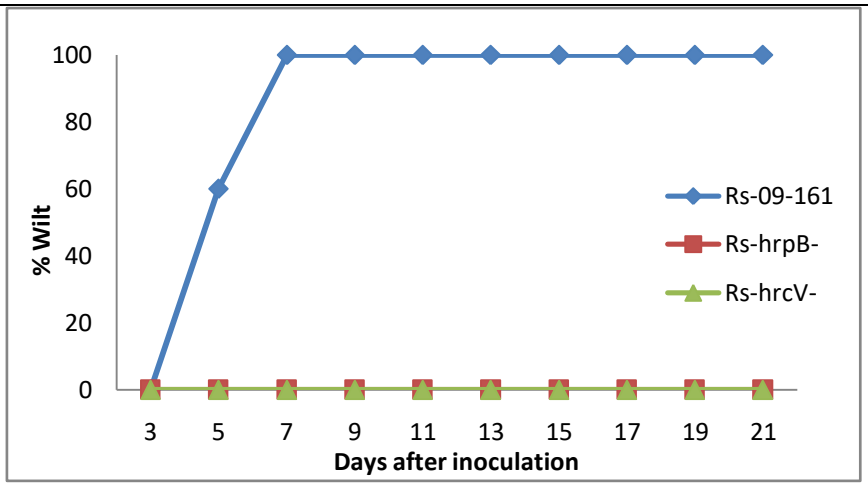
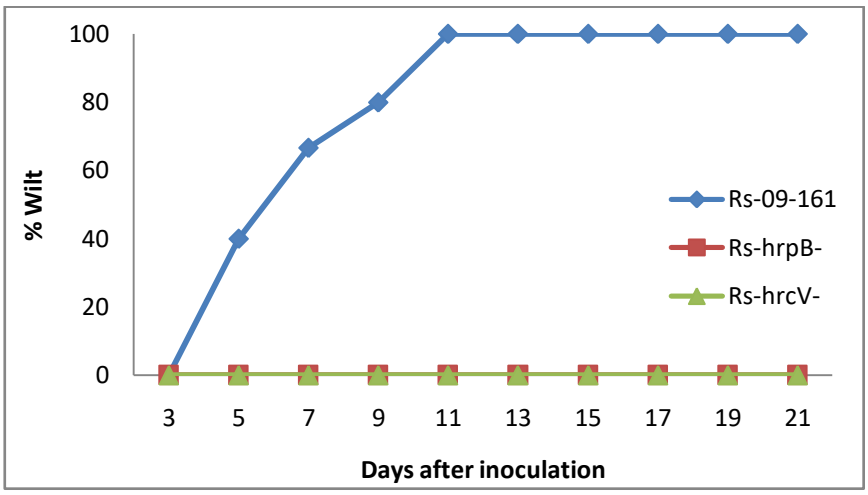


Fig 5.1: Bacterial wilt incidence in brinjal (*Agassaim*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T3SS mutants: Rs-hrpB^{-} and Rs-hrcV^{-}, by soil drench inoculation (A) Experiment 1 conducted with 2 replications (B) Experiment 2 conducted with 3 replications (C) Wilt % obtained as mean of Experiment 1 and 2. The plants were observed for 21 DPI.

A



B



C

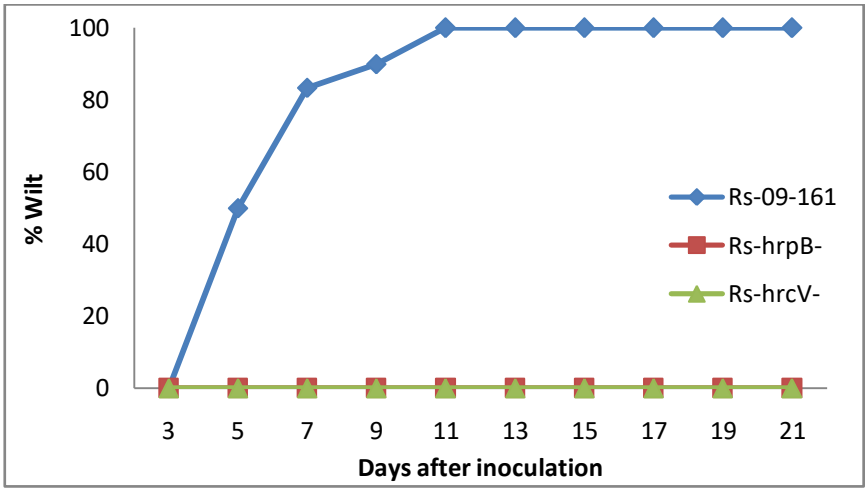


Fig. 5.2. Bacterial wilt incidence in tomato (*Pusa Ruby*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T3SS mutants: Rs-hrpB- and Rs-hrcV- on tomato by soil drench inoculation. (A) Experiment 1 conducted with 3 replications (B) Experiment 2 conducted with 3 replications (C).Wilt % obtained as mean of Experiment 1 and 2. The plants were observed for 21 DPI.

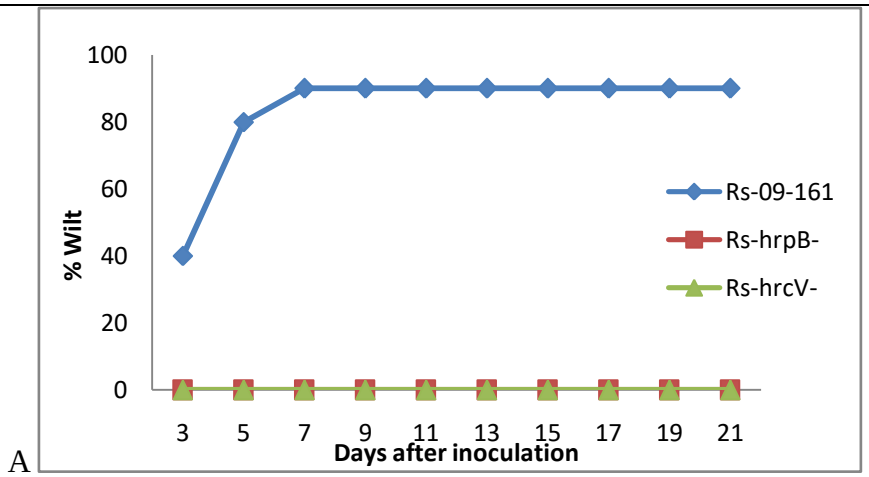
5.3.3.1.2 Petiole inoculation

Susceptible brinjal cultivar –*Agassaim*

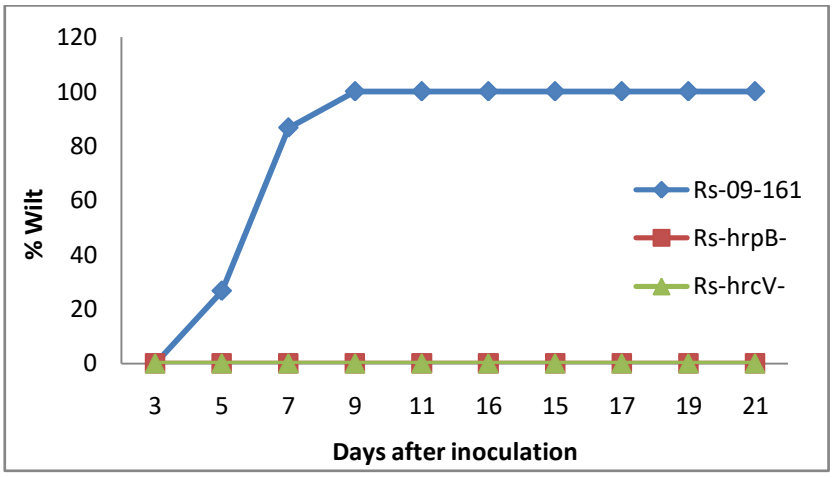
The bacterial wilt incidence by cut petiole inoculation method was assayed on brinjal (cv., *Agassaim*) in two different experiments. The wilt incidence exhibited by T3SS mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$, is depicted in Fig 5.3. In brinjal 95% wilt was exhibited by Rs-09-161 on eighth DAI. In experiment 1, 40% wilt incidence was observed on 3 DAI and increased to 90% on 7 DAI, whereas in experiment 2, wilt was initiated on 4 DAI and reached 100% on 9 DAI. The mutants were non-pathogenic on susceptible brinjal in both the experiments.

Susceptible tomato variety –*Pusa Ruby*

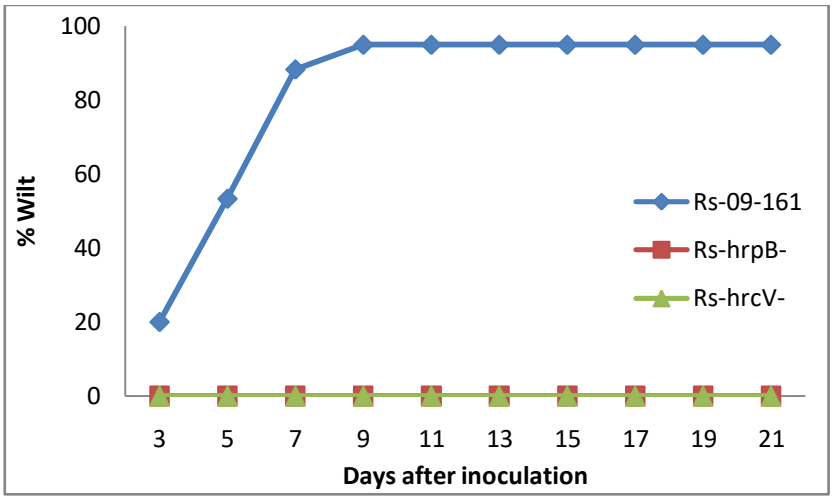
The bacterial wilt incidence by cut petiole inoculation method was assayed on tomato (var., *Pusa Ruby*) in three different experiments with two replications in Experiment 1, 2 and three replications in Experiment 3. The T3SS mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$ were non-pathogenic on susceptible tomato whereas the wild type strain Rs-09-161 exhibited 100% wilt within 10 DAI in the individual experiments (Fig. 5.4).



A



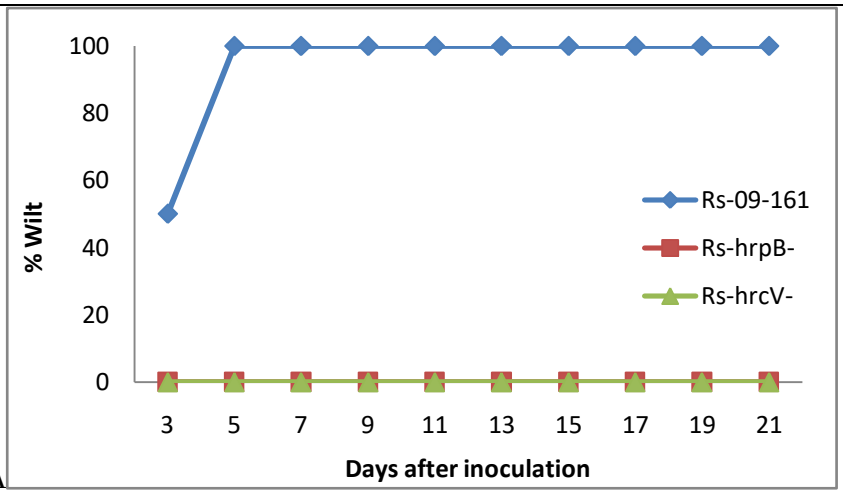
B



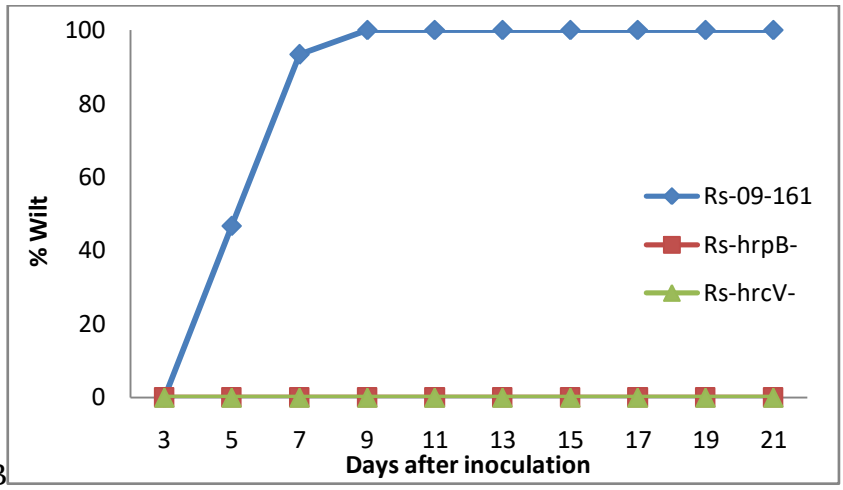
C

Fig. 5.3. Bacterial wilt incidence in brinjal (*Agassaim*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T3SS mutants: Rs-hrpB- and Rs-hrcV-, by cut petiole inoculation (A) Experiment 1 conducted with 2 replications (B) Experiment 2 conducted with 3 replications (C) Wilt % obtained as mean of Experiment 1 and 2. The plants were observed for 21 DPI.

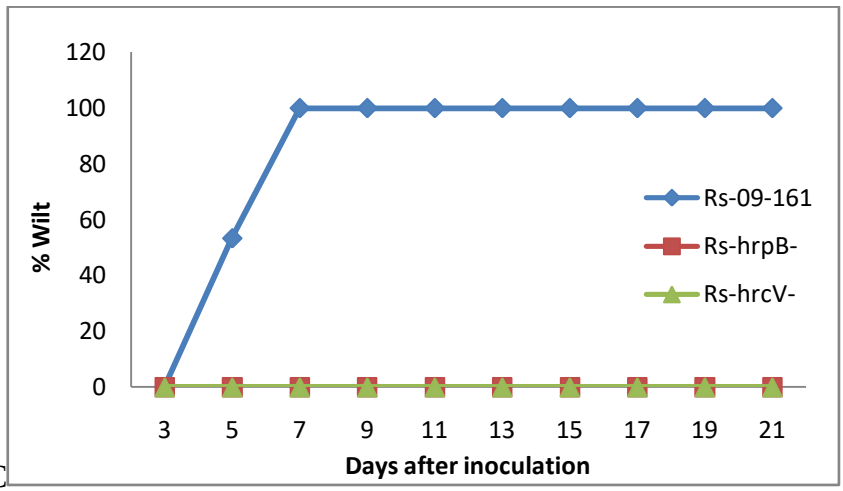
A


100
80
60
40
20
0
% Wilt
3
5
7
9
11
13
15
17
19
21
Days after inoculation
Rs-09-161
Rs-hrpB-
Rs-hrcV-

B


100
80
60
40
20
0
% Wilt
3
5
7
9
11
13
15
17
19
21
Days after inoculation
Rs-09-161
Rs-hrpB-
Rs-hrcV-

C


120
100
80
60
40
20
0
% Wilt
3
5
7
9
11
13
15
17
19
21
Days after inoculation
Rs-09-161
Rs-hrpB-
Rs-hrcV-

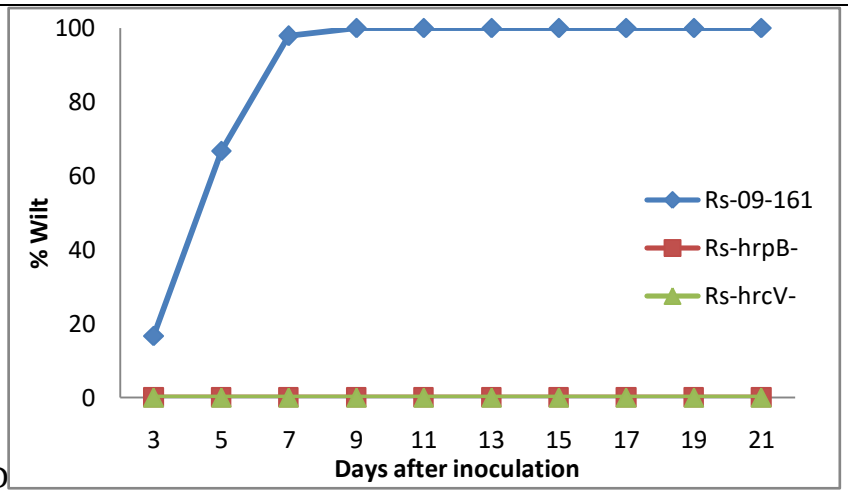
D 

Fig.5.4. Bacterial wilt incidence in tomato (*Pusa Ruby*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T3SS mutants on tomato by cut petiole inoculation. (A) Experiment 1 conducted with 2 replications. (B) Experiment 2 conducted with 3 replications. (C) Experiment 3 conducted with 3 replications. (D) Wilt % obtained as mean of Experiment 1, 2 and 3. The plants were observed for 21 DPI.

It is well-established that the T3SS in *R. solanacearum* is the main pathogenicity determinant and the mutants of the T3SS are unable to cause the disease (Boucher *et al*., 1988; Salanoubat *et al.*, 2002). Our results are in agreement with this observation as we did not observe any wilt in the brinjal and tomato seedlings inoculated with the mutant Rs-hrcV^{-} and tomato seedlings inoculated with Rs-hrpB^{-}. However, the brinjal seedlings inoculated by drench inoculation depicted an average of 3.33% wilt. This average wilt is a mean of 2 experiments conducted separately. The soil drench inoculation follows the conditions of natural infection process and petiole inoculation includes introduction of the pathogen into the vascular system of the seedlings. The wilt incidence caused by the T3SS mutants on tomato and brinjal are depicted in Fig. 5.5.

HrpB is the transcriptional regulator which controls the expression of T3SS and the T3Es (Genin and Denny, 2012) and *HrcV* is a trans membrane protein that forms a component of hrp Pili, the needle like apparatus which is involved in translocation of the T3Es into

the host cells. The *hrpB* mutant of *R. solanacearum* will fail to express the *hrpB*-dependent T3Es whereas the mutant of *hrcV* gene will fail to secrete the T3Es in the external environment. These factors make them excellent choice of gene for development of T3SS mutant.

(A)  (B) 

Fig.5.5. In plants assays using *R. solanacearum* strains Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$. A: Tomato seedlings (*Pusa Ruby*) inoculated on the petiole with Rs-hrpB$^-$ and Rs-09-161, Rs-09-161 inoculated plants show complete wilt whereas Rs-hrpB$^-$ and Rs-hrcV$^-$ inoculated plants do not display any wilt symptom. B: Susceptible brinjal cultivar *Agassaim* inoculated by drench inoculation, Rs-09-161 displays wilt symptoms whereas no wilt is observed with plants inoculated with Rs-hrpB$^-$ and Rs-hrcV$^-$.

The expression of the T3SS was considered very crucial during the early stages of infection to suppress the host immunity and gets turned down in the higher cell densities (Genin *et al.*, 2005; Mole *et al.*, 2007). However, the recent studies using *in planta* transcriptome have proved that the T3SS plays an important role throughout the disease cycle and is not repressed during high cell densities (Jacobs *et al.*, 2012; Monteiro *et al.*, 2012b). This can be the probable reason for the reduction in wilt by the Rs-hrpB$^-$ and Rs-hrcV$^-$ mutants in tomato and brinjal seedlings inoculated through drench and petiole inoculation.

5.3.3.1.3. Colonization of T3SS mutants in brinjal

The colonization ability of the mutants was highly reduced in brinjal seedlings as compared to the wild type strain even though the inoculum concentration of the three strains was uniform.

In the plant colonization experiment, experiment 1 was conducted to standardize the optimum day for studying the colonization of wild type and mutants of *R. solanacearum*. Day four was considered as optimum day for colonization studies as the wild type showed an average population of 11 log CFU.g^{-1} and Rs-hrpB^{-} also showed its highest population. The population of wild type Rs-09-161 in the roots of the susceptible brinjal was 6 log CFU.g^{-1} on day two which gradually increased to 14 log CFU.g^{-1} till day six and in the stem was 4 log CFU.g^{-1} on day two and increased to 16 log CFU.g^{-1} on day eight in experiment 1. On day 10, the wild type inoculated plants had wilted completely so the colonisation was not assessed for them. Rs-hrpB^{-} did not colonise in the susceptible plants and the population was 1.2 log CFU.g^{-1}on day two, 3.3 log CFU.g^{-1} on day four and 1.5 log CFU.g^{-1} on day 10 in the roots and 1.2 log CFU.g^{-1} on day 10 in the stem respectively in experiment 1. After day four, the population of Rs-hrpB^{-} disappeared which might be due to the reduced virulence mechanism of the Rs-hrpB^{-} mutant against the host. Analysis of the infection process of wild type *R solanacearum* and its *hrpB* mutant in tomato seedlings through bioluminescence demonstrated that the *hrpB* mutant does not overcome the host defense during invasion of the root and the hypocotyl tissue (Matsuda *et al.*, 2000). The reduction in colonization was also observed in *In planta* assays on tomato, brinjal and *Arabidopsis thaliana* with wild type and hrp- mutant of *R. solanacearum* (Monteiro *et al.*, 2012a).

In experiment two we observed that, the mutants, Rs-hrpB$^-$ and Rs-hrcV$^-$ failed to colonize in the plants and their population was less than 8 log CFU.g^{-1} which is correlated as threshold population for initiating wilt in the susceptible tomato (Huang and Allen, 2000) whereas the wild type attained the population of 10 log CFU.g^{-1}(Table 5.4). Similar results were obtained by Vasse and co-workers (2000) who revealed that the *hrpB* and *hrcV* mutants were poorly infective and weakly invasive, and observed limited multiplication in the intercellular spaces of the root cortex and vascular system of the inoculated tomato plants.

Table 5.4.Plant colonisation by *R. solanacearum* isolates Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$

		Population of *R. solanacearum* (Log)					
		Expt 1					Expt 2
Strain	Sample	day2	day 4	day 6	day 8	day 10	day 4
Rs-09-161	root	6.00	11.25	14.27	ND	ND	10.18
	stem	4.11	8.89	14.29	16.58	ND	ND
Rs-hrpB-	root	1.21	3.38	0	0	1.50	5.74
	stem	0	0	0	0	1.28	ND
Rs-hrcV-	root	ND	ND	ND	ND	ND	5.65
	stem	ND	ND	ND	ND	ND	ND

The data obtained is a mean of 3 samples with two replications. ND: Not done

5.3.3.2 T6SS mutants of *R. solanacearum*

The results obtained in the analysis of T6SS mutants are as follows:

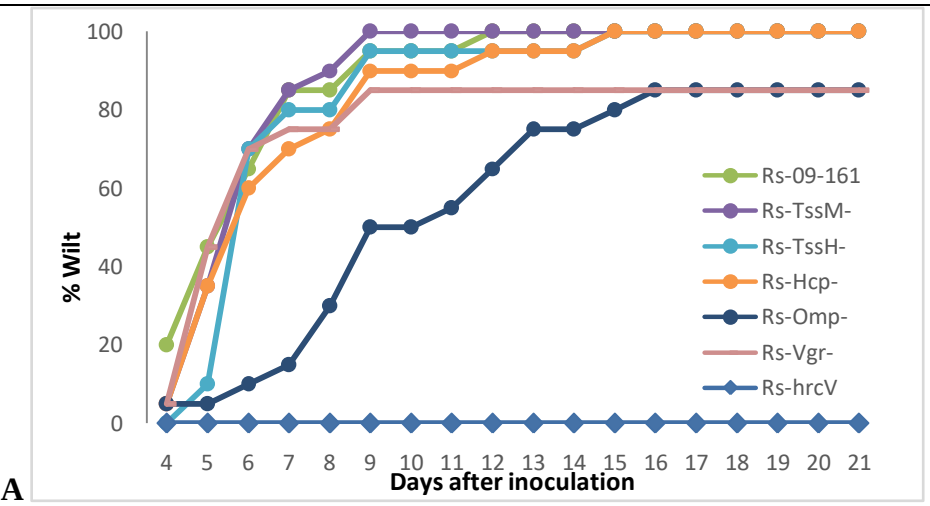
5.3.3.2.1 Soil drench inoculation

Susceptible tomato variety-*Arka Vikash*

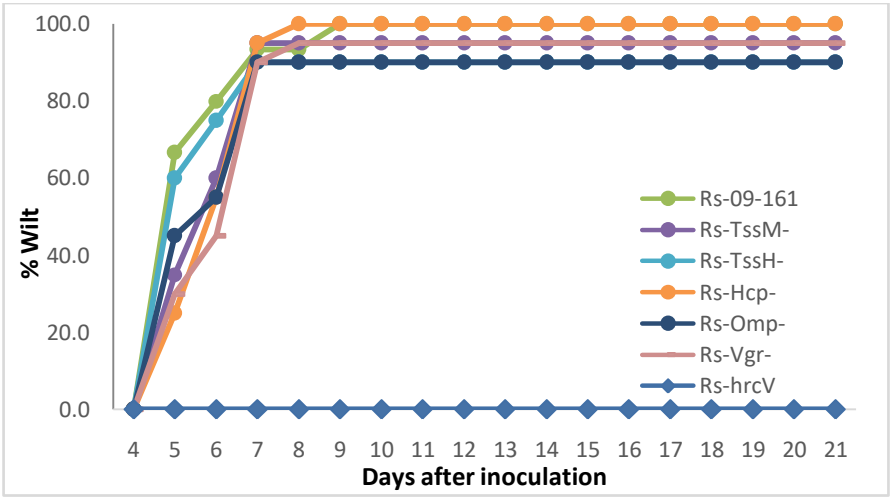
The virulence of *R. solanacearum* T6SS mutants *viz.* Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$ was tested by soil drench inoculation on susceptible tomato (var., *Arka Vikash*). In experiment 1, wilt incidence was observed on fourth DAI in Rs-09-161 and in majority of the T6SS mutants except in Rs-TssH$^-$. Wild type strain caused 100% wilt on 12 DAI along with Rs-TssM$^-$. In case of Rs-TssH$^-$ and Rs-Hcp$^-$, 95% wilt incidence was observed on day 12. The least wilt incidence was observed in Rs-OmpA$^-$ (65%) followed by Rs-VgrG3$^-$ (85%). At the end of experiment, on day 21, 85% of wilt incidence was observed in both Rs-OmpA$^-$ and Rs-VgrG3$^-$ (Fig. 5.6a).

In experiment 2, wilt incidence was initiated on fifth DAI in wild type as well as all five T6SS mutants. Wild type caused 100% wilt incidence on ninth DAI. The least wilt incidence was observed in Rs-TssH$^-$ and Rs-OmpA$^-$ (90%) followed by Rs-TssM$^-$ and Rs-VgrG3$^-$(95%) (Fig 5.6b). The graph obtained by combining all the replications is depicted in fig 5.6. Pooled data indicated that the wild type caused more than 50% wilt on fifth DAI, whereas in the mutants wilt was in the range of 25-38%. Wild type caused 100% wilt 12 DAI and the lowest among the mutants on day 12 was Rs-OmpA$^-$ (77.5%) followed by Rs-VgrG3$^-$ (90%). No wilt was observed in Rs-hrcV$^-$ (Fig. 5.6C and Table 5.5).

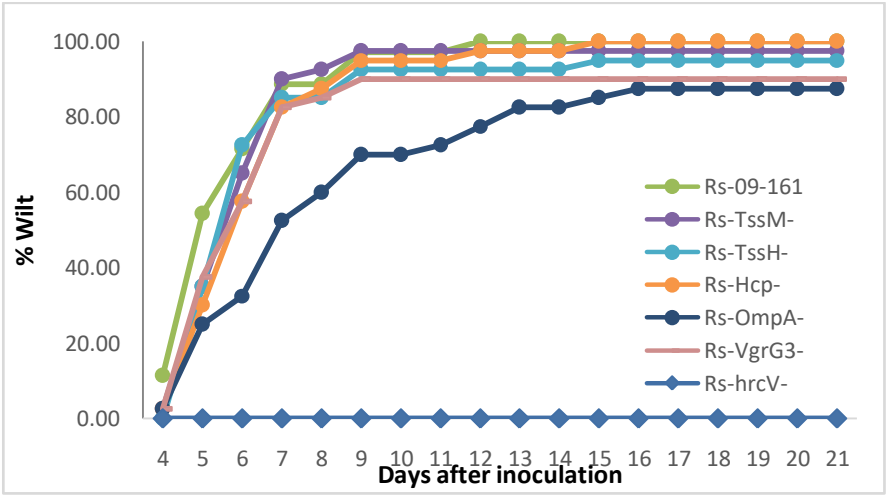
Incidence of wilt by wild type and T6SS mutants were compared on seventh DAI. From earlier studies it is understood that incidence of wilt after seven DAI provides fair idea about the virulence of the isolate. On the seventh day, wild type caused a wilt incidence of 88.6%whereas Rs-OmpA$^-$ caused significant reduction in wilt incidence (52.5%).



A



B



C

5.6 Bacterial wilt incidence in tomato (*Arka Vikash*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T6SS mutants by soil drench inoculation. (A) Experiment 1 conducted with 4 replications (B) Experiment 2 conducted with 4 replications (C) Mean wilt % obtained by combining all replications. The plants were observed for 21 DPI.

Table 5.5.Wilt incidence caused by the T6SS mutants on susceptible tomato (var., *Arka Vikash*) by soil drench inoculation.

	Mean wilt %																	
DAI	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rs-09-161	11.4	54.3 ^{a}	71.4 ^{a}	88.6 ^{a}	88.6 ^{a}	97.1 ^{a}	97.1 ^{a}	97.1 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}
Rs-TssM^{-}	2.5	35.0 ^{ab}	65.0 ^{a}	90.0 ^{a}	92.5 ^{a}	97.5 ^{a}	97.5 ^{a}	97.5 ^{a}	97.5 ^{a}	97.5 ^{a}	97.5	97.5 ^{ab}	97.5 ^{ab}	97.5 ^{ab}	97.5 ^{ab}	97.5 ^{ab}	97.5 ^{ab}	97.5 ^{ab}
Rs-TssH^{-}	0	35.0 ^{ab}	72.5 ^{a}	85.0 ^{a}	85.0 ^{a}	92.5 ^{a}	92.5 ^{a}	92.5 ^{a}	92.5 ^{a}	92.5 ^{ab}	92.5	95.0 ^{ab}	95.0 ^{ab}	95.0 ^{ab}	95.0 ^{ab}	95.0 ^{ab}	95.0 ^{ab}	95.0 ^{ab}
Rs-Hcp^{-}	2.5	30.0 ^{ab}	57.5 ^{a}	82.5 ^{a}	87.5 ^{a}	95.0 ^{a}	95.0 ^{a}	95.0 ^{a}	97.5 ^{a}	97.5 ^{a}	97.5	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}
Rs-OmpA^{-}	2.5	25.0 ^{bc}	32.5 ^{b}	52.5 ^{b}	60.0 ^{b}	70.0 ^{b}	70.0 ^{b}	72.5 ^{b}	77.5 ^{b}	82.5 ^{b}	82.5 ^{b}	85.0 ^{b}	87.5 ^{b}	87.5 ^{b}	87.5 ^{b}	87.5 ^{b}	87.5 ^{b}	87.5 ^{b}
Rs-VgrG3^{-}	2.5	37.5 ^{ab}	57.5 ^{a}	82.5 ^{a}	85.0 ^{a}	90.0 ^{a}	90.0 ^{a}	90.0 ^{a}	90.0 ^{ab}	90.0 ^{ab}	90	90.0 ^{ab}	90.0 ^{ab}	90.0 ^{ab}	90.0 ^{ab}	90.0 ^{ab}	90.0 ^{ab}	90.0 ^{ab}
Rs-hrcV^{-}	0	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}
CD (0.05)	NS	25.14	23.9	22.9	20.9	16.07	16.07	16.35	14.7	13.2	13.2	13.3	12.1	12.1	12.1	12.1	12.1	12.1
CD (0.01)	NS	33.61	32	30.6	27.97	21.49	21.49	21.86	19.6	17.7	17.7	17.8	16.18	16.18	16.18	16.18	16.18	16.18

Data obtained is a mean of 8 replications. Each replication consisted of 5 plants. The plants were observed for 21 DPI. NS: Non significant

In a column, means followed by the same letter are not significantly different at 5%.

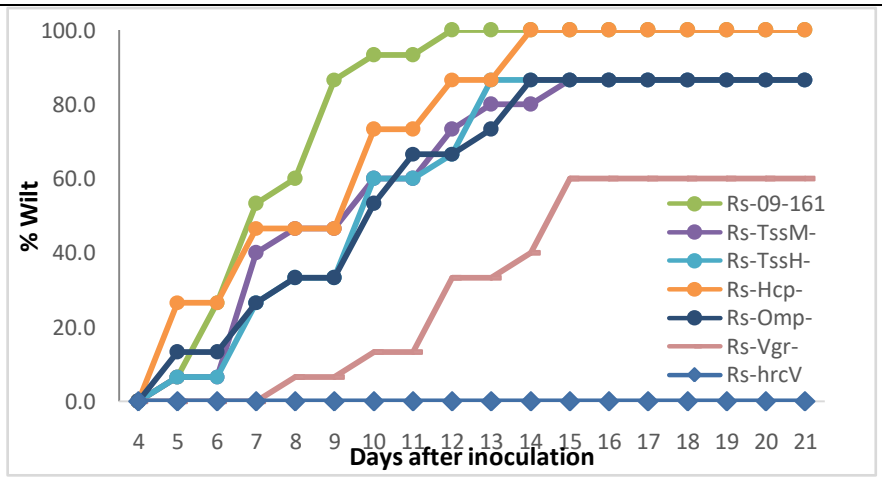
Susceptible brinjal cultivar –*Agassaim*

The virulence of *R. solanacearum* T6SS mutants: Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$ was tested by soil drench inoculation on susceptible brinjal (cv., *Agassaim)* in two different experiments

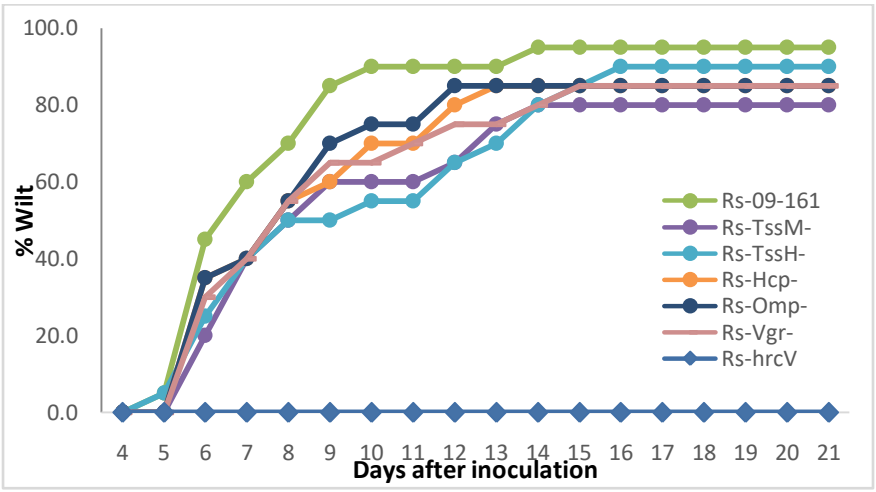
In experiment 1, wilt incidence was initiated on fifth DAI in wild type and in majority of the T6SS mutants, except in Rs-VgrG3$^-$. Wild type caused more than 50% wilt incidence on seventh DAI whereas in Rs-TssH$^-$ and Rs-OmpA$^-$ wilt incidence was limited to 27% and was not observed in Rs-VgrG3$^-$. Wild type caused 100% wilt incidence on 12 DAI. But in the T6SS mutants it was limited to less than 87%. In case of Rs-VgrG3$^-$, wilt incidence was 33% while that of Rs-TssH$^-$ and Rs-OmpA$^-$ it was 67%. At the end of the experiment, Rs-VgrG3$^-$ caused the lowest wilt incidence (60%) (Fig.5.7a). In experiment 2, wilt incidence was observed in wild type on fifth DAI and in the T6SS mutants it was delayed by one day. Wild type caused 60% wilt incidence on seven DAI whereas in the T6SS mutants it was limited to 40%. Wild type caused a highest of 95% wilt incidence on 14 DAI. In case of T6SS mutants, the wilt incidence was less than 86% on day 14 (Fig.5.7b).

The pooled mean values of the wilt incidence are represented in (Fig.5.7c and Table 5.6). In soil drench inoculation, wilting of plants was initiated on five DAI except in Rs-VgrG3$^-$. Wild type caused more than 50% wilt on seventh DAI, whereas in the mutants, it ranged from 23-40%. Wild type caused a maximum wilt of 97.14% on 14 DAI and the lowest among the mutants was Rs-VgrG3$^-$ (62.9%). Based on the seventh day data, Rs-TssH$^-$ (34.2%), Rs-OmpA$^-$ (34.2%) and Rs-VgrG3$^-$ (22.8%) significantly reduced the wilt incidence as compared to the wild type (57.1%).

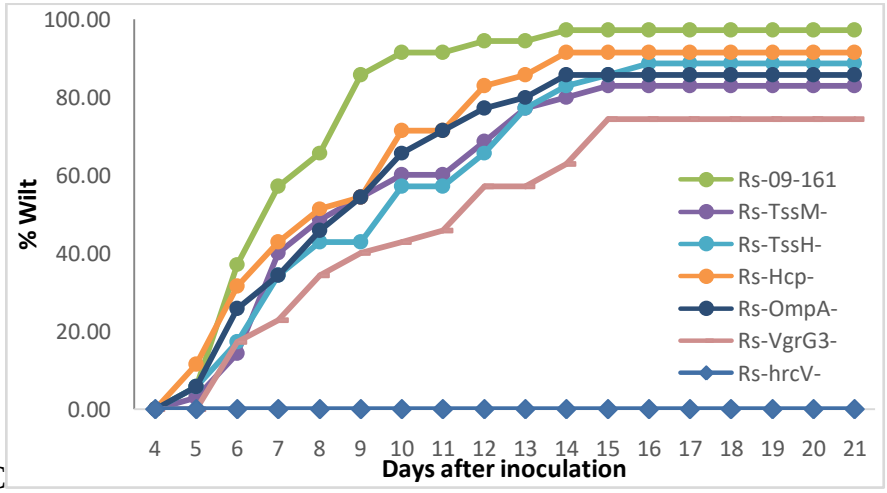
A



B



C



5.7. Bacterial wilt incidence in brinjal (cv., *Agassaim*) inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T6SS mutants by soil drench inoculation. (A) Experiment 1 conducted with 3 replications (B) Experiment conducted with 4 replications (C) Wilt % obtained by combining all replications. The plants were observed for 21 DPI.

Table 5.6.Wilt incidence caused by the T6SS mutants^{-} in susceptible brinjal (cv., *Agassaim*) by soil drench inoculation.

DAI	Mean wilt %																	
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rs-09-161	0	5.7	37.1	57.1^{a}	65.7^{a}	85.7^{a}	91.4^{a}	91.4^{a}	94.2^{a}	94.2^{a}	97.1^{a}	97.1^{a}	97.1^{a}	97.1^{a}	97.1^{a}	97.1^{a}	97.1^{a}	97.1^{a}
Rs-TssM^{-}	0	2.8	14.2	40.0^{ab}	48.5^{ab}	54.2^{b}	60.0^{bc}	60.0^{b}	68.5^{bc}	77.1^{ab}	80.0^{ab}	82.8^{ab}	82.8^{ab}	82.8^{ab}	82.8^{ab}	82.8^{ab}	82.8^{ab}	82.8^{ab}
Rs-TssH^{-}	0	5.7	17.1	34.2^{b}	42.8^{b}	42.8^{b}	57.1^{bc}	57.1^{b}	65.7^{bc}	77.1^{ab}	82.8^{ab}	85.7^{ab}	88.5^{ab}	88.5^{ab}	88.5^{ab}	88.5^{ab}	88.5^{ab}	88.5^{ab}
Rs-Hcp^{-}	0	11.4	31.4	42.8^{ab}	51.4^{ab}	54.2^{b}	71.4^{ab}	71.4^{ab}	82.8^{ab}	85.7^{a}	91.4^{a}	91.4^{ab}	91.4^{ab}	91.4^{ab}	91.4^{ab}	91.4^{ab}	91.4^{ab}	91.4^{ab}
Rs-OmpA^{-}	0	5.7	25.7	34.2^{b}	45.7^{ab}	54.2^{b}	65.7^{ab}c	71.4^{ab}	77.1^{abc}	80.0^{ab}	85.7^{a}	85.7^{ab}	85.7^{ab}	85.7^{ab}	85.7^{ab}	85.7^{ab}	85.7^{ab}	85.7^{ab}
Rs-VgrG3^{-}	0	0	17.1	22.8^{b}	34.2^{b}	40.0^{b}	42.8^{c}	45.7^{b}	57.1^{c}	57.1^{b}	62.8^{b}	74.2^{b}	74.2^{b}	74.2^{b}	74.2^{b}	74.2^{b}	74.2^{b}	74.2^{b}
Rs-hrcV^{-}	0	0	0	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{c}	0.0^{d}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.00^{c}	0.0^{c}	0.00^{c}	0.0^{c}	0.0^{c}
CD (0.05)	0	22.11	14.655	22.11	22.2	24.21	25.99	26.45	25.49	24.67	20.98	17.97	17.97	17.97	17.97	17.97	17.97	17.97
CD (0.01)	0	29.65	19.648	29.65	29.76	32.46	34.85	35.46	34.18	33.07	28.12	24.09	24.09	24.09	24.09	24.09	24.09	24.09

Data obtained is a mean of 7 replications. Each replication consisted of 5 plants. The plants were observed for 21 DPI.The plants were observed for 21 DPI. NS: Non significant in a column, means followed by the same letter are not significantly different at 5%

5.3.3.2.2 Petiole inoculation

Susceptible tomato variety-*Arka Vikash*

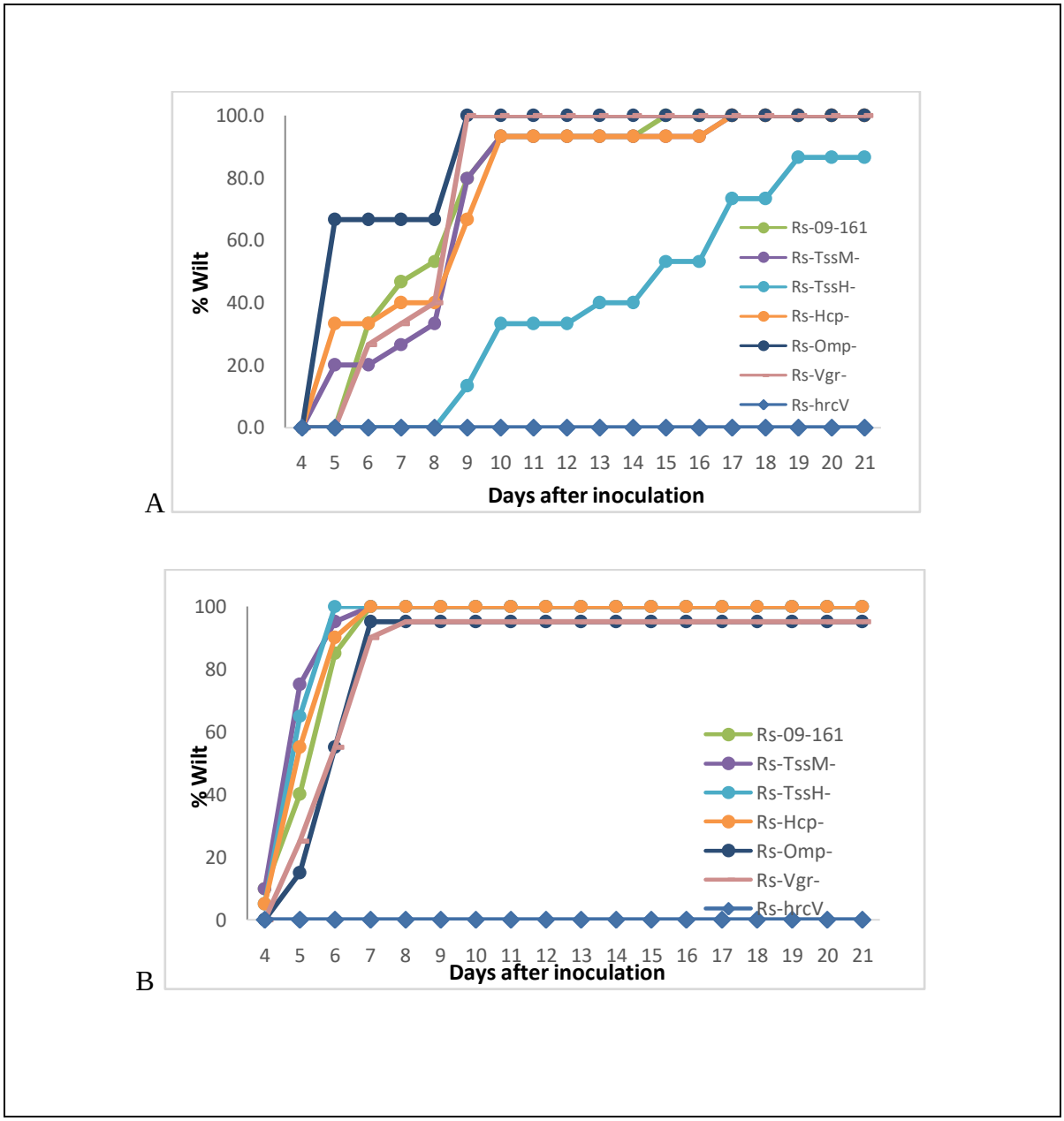
The virulence of *R. solanacearum* T6SS mutants was tested on susceptible tomato (var., *Arka Vikash*) in four different experiments with Rs-hrcV$^-$ as a negative control.

In experiment 1, wilt incidence was initiated on five DAI in Rs-Hcp$^-$ and Rs-OmpA$^-$ whereas in the wild type it was observed on sixth DAI. Wild type caused more than 50% wilt on eighth DAI. In Rs-TssH$^-$ the wilt incidence was not observed and in case of Rs-TssM$^-$ it was limited to less than 34%. Wild type caused 100% wilt incidence on 15 DAI and in case of Rs-TssH$^-$ it was reduced to 53% (Fig. 5.8a). In experiment 2, wilt was observed only in wild type and Rs-TssM$^-$ on fourth DAI. Wild type caused 100% wilt on seven DAI. On day seven, lowest wilt incidence was observed in Rs-VgrG3$^-$ (90%) followed by Rs-OmpA$^-$ (95%) (Fig. 5.8b).

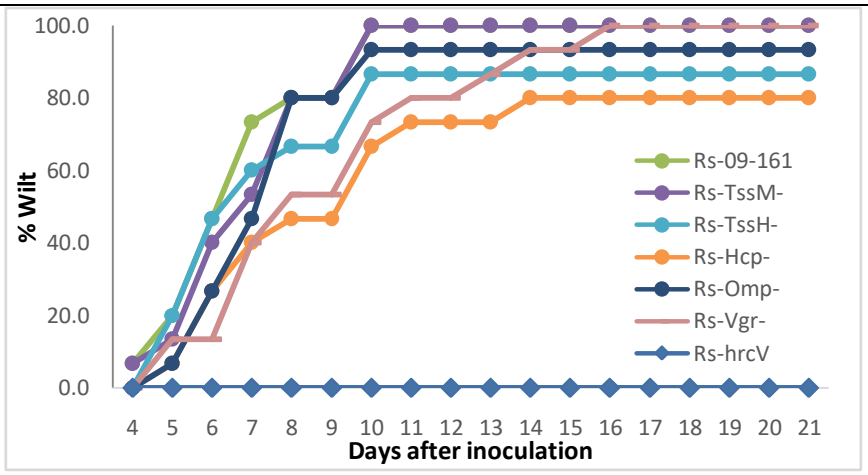
In experiment 3, wilt incidence was observed in wild type along with Rs-TssM$^-$ on fourth DAI. More than 50% wilt was observed in wild type on seven DAI. In case of the T6SS mutants Rs-OmpA$^-$, Rs-Hcp$^-$ and Rs-VgrG3$^-$ wilt incidence was less than 47%. Wild type caused 100% wilt on tenth DAI but, was limited to less than 74% in Rs-Hcp$^-$ and Rs-VgrG3$^-$(Fig.5.8c). In experiment 4, wilt incidence was observed on fourth DAI. In the wild type wilt incidence was 40% whereas in Rs-VgrG3$^-$ it was limited to 7%. Wild type caused 100% wilt incidence on sixth DAI. The lowest wilt incidence was observed in Rs-VgrG3$^-$ (27%) followed by Rs-OmpA$^-$ (67%) (Fig.5.8d).

The wilt incidence caused by the T6SS mutants on *Arka Vikash* in individual experiments and by combining all the replications is represented in Fig. 5.8 and Table 5.7. Analysis of the pooled data indicated that wilting of plants was observed on fourth DAI. Wild type caused more than 50% wilt on six DAI. Rs-VgrG3$^-$ caused 32% wilt on six DAI while in

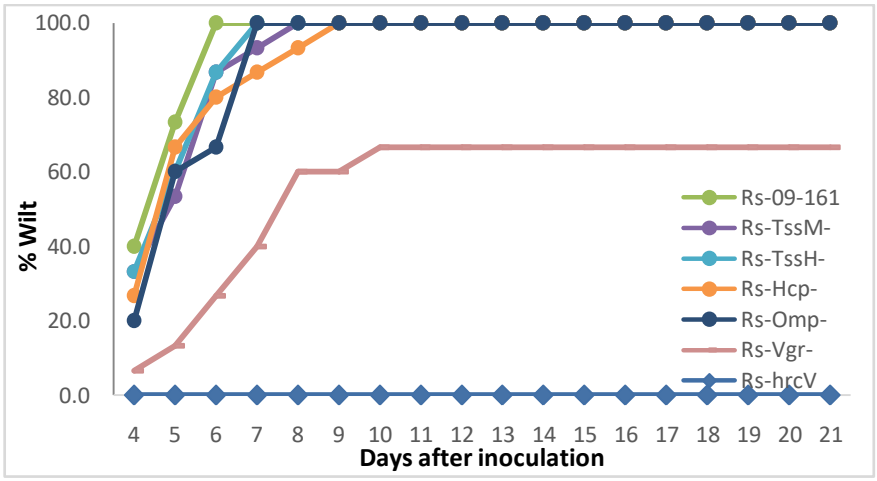
remaining type six mutants the wilt ranged from 54-63%. Wild type caused 100% wilt on 15 DAI. The lowest wilt was observed in Rs-TSSH$^-$ (86.15%) and Rs-VgrG3$^-$ (89.23%). Based on the seventh day data, Rs-VgrG3$^-$ significantly reduced the wilt incidence (53.9%). Other mutants *viz.* Rs-TssH$^-$ (67.7%), Rs-Hcp$^-$ (69.2%) and Rs-TssM$^-$ (70.8%) also reduced the wilt incidence compared to the wild type (81.5%).



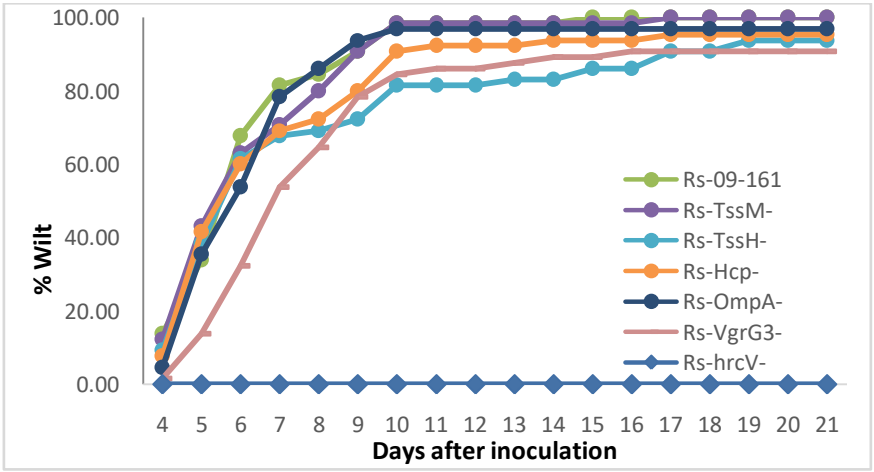
C



D



E



5.8. Bacterial wilt incidence in tomato (*Arka Vikash*) inoculated with *R. solanacearum* wild type strain Rs-09-161 and its T6SS mutants on tomato by cut petiole inoculation. (A) Experiment 1 conducted with 3 replications (B) Experiment 2 conducted with 4 replications **(**C) Experiment 3 conducted with 3 replications (D) Experiment 4 conducted with 3 replications (E) Graph obtained by combining mean of all replications. The plants were observed for 21 DPI.

Table 5.7.Wilt incidence caused by the T6SS mutants in susceptible in tomato (var., *Arka Vikash*) by cut petiole inoculation.

DAI	Mean wilt %																	
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rs-09-161	13.9 ^{a}	33.9^{ab}	67.7^{a}	81.5^{a}	84.6^{a}	90.8^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Rs-TssM^{-}	12.3 ^{ab}	43.1^{a}	63.1^{a}	70.8 ^{ab}	80.0^{ab}	90.8^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	98.5^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Rs-TssH^{-}	9.2 ^{abc}	38.5^{a}	61.5^{a}	67.7 ^{ab}	69.2^{ab}	72.3^{b}	81.5^{b}	81.5^{b}	81.5^{b}	83.1^{b}	83.1^{a}	86.2^{a}	86.2^{a}	90.8^{a}	90.8^{a}	93.9^{a}	93.9^{a}	93.9^{a}
Rs-Hcp^{-}	7.7 ^{abcd}	41.5^{a}	60.0^{a}	69.2 ^{ab}	72.3^{ab}	80.0 ^{ab}	90.8^{ab}	92.3^{ab}	92.3^{ab}	92.3^{ab}	93.9^{a}	93.9^{a}	93.9^{a}	95.4^{a}	95.4^{a}	95.4^{a}	95.4^{a}	95.4^{a}
Rs-OmpA^{-}	4.6 ^{bcd}	35.4^{ab}	53.9 ^{a}	78.5 ^{a}	86.2^{a}	93.9^{a}	96.9^{ab}	96.9^{a}	96.9^{a}	96.9^{ab}	96.9^{a}	96.9^{a}	96.9^{a}	96.9^{a}	96.9^{a}	96.9^{a}	96.9^{a}	96.9^{a}
Rs-VgrG3^{-}	1.5 ^{cd}	13.9^{bc}	32.3^{b}	53.9 ^{b}	64.6^{b}	78.5^{ab}	84.6^{ab}	86.2^{ab}	86.2^{ab}	87.7^{ab}	89.2^{a}	89.2^{a}	90.8^{a}	90.8^{a}	90.8^{a}	90.8^{a}	90.8^{a}	90.8^{a}
Rs-hrcV^{-}	0.0 ^{d}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0c	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}
CD (0.05)	8.54	21.9	21.21	21.2	19.8	18.2	16.13	14.9	14.9	13.8	13.5	12.2	12.2	11.2	11.2	11.2	11.2	11.2
CD (0.01)	NS	29.1	28.16	8.16	26.3	24.2	21.4	19.9	19.9	18.3	18	16.2	16.1	14.9	14.9	14.9	14.9	14.9

Data obtained is a mean of 13 replications. Each replication consisted of 5 plants. The plants were observed for 21DPI. NS: Non significant

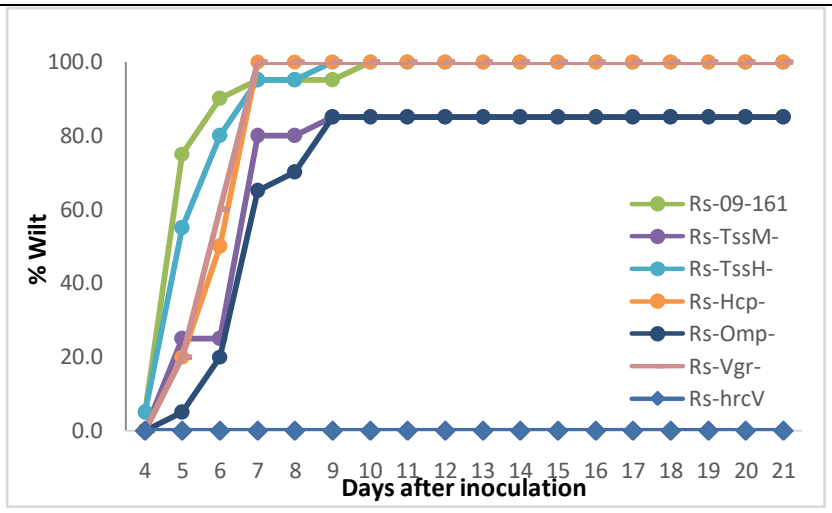
In a column, means followed by the same letter are not significantly different at 1%.

Susceptible brinjal cultivar –*Agassaim*

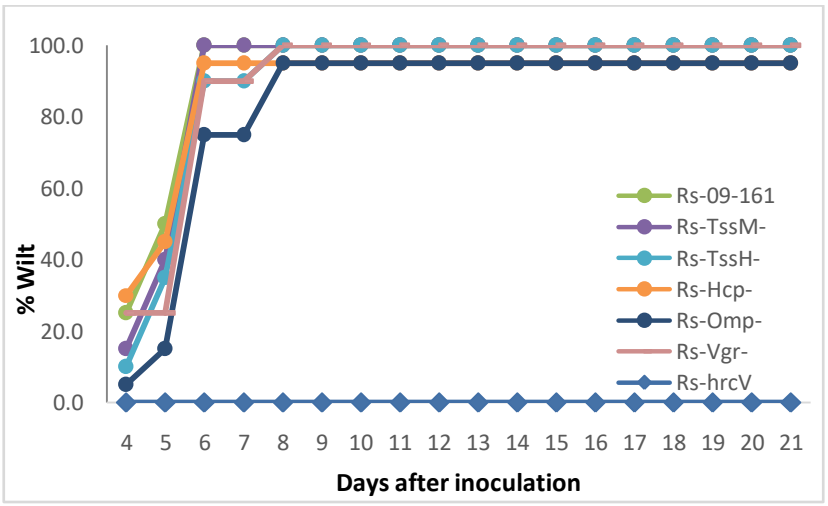
The virulence of *R. solanacearum* T6SS mutants was tested on susceptible brinjal (cv., *Agassaim)*. In experiment 1, wilt incidence was observed in wild type and T6SS mutant Rs-TssH$^-$ on fourth DAI. The initiation of wilt incidence was delayed to fifth day in Rs-TssM$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$.Wild type caused 100% wilt incidence on tenth DAI whereas, in Rs-TssM$^-$ and Rs-OmpA$^-$ it was limited to 85% till the end of the experiment (Fig. 5.9a).

In experiment 2, the wilt incidence was observed on day four in wild type as well as all T6SS mutants. Fifty percent wilt incidence was observed on day five in wild type and reduced incidence (15-35%) was observed in Rs-VgrG3$^-$, Rs-OmpA$^-$ and Rs-TssH$^-$. Wild type caused 100% wilt incidence on day six and the lowest among the mutants was Rs-OmpA$^-$ (75%) followed by Rs-TssH$^-$ and Rs-VgrG3$^-$ (90%)(Fig. 5.9b).

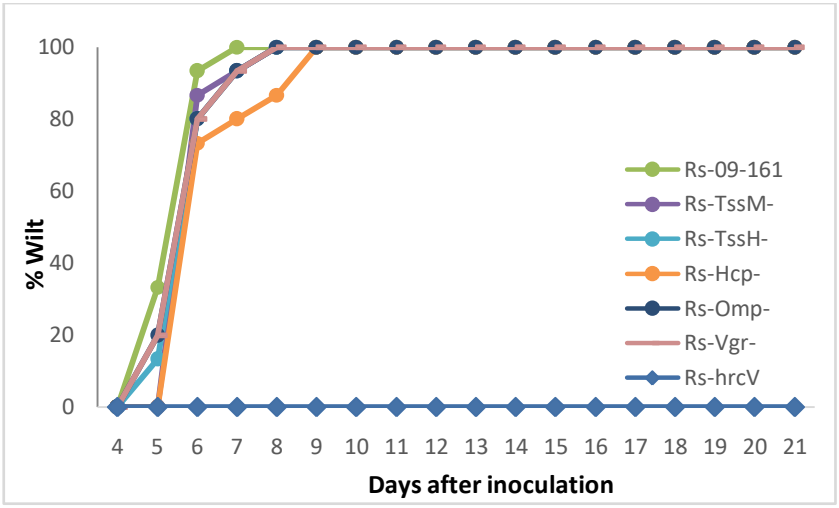
In experiment 3, wild type caused 100% wilt incidence on seventh DAI while in the T6SS mutants it was less than 94% and the lowest was observed in Rs-Hcp$^-$ (80%)(Fig. 5.9c). The mean values for the wilt incidence obtained by combining the replications are represented in Fig 5.9 and Table 5.8. Analysis of the pooled data indicated that wilting was observed on 4 DAI. Wild type caused more than 50% wilt on fifth DAI, whereas, in the mutants it ranged from 22-36%. Wild type caused 100% wilt 10 DAI and the lowest among the mutants was Rs-OmpA$^-$ (92.7%) followed by Rs-TssM$^-$ (94.5%). On the seventh day, Rs-OmpA$^-$ showed a significant reduction in wilt (76.4%) incidence whereas wild type caused 98.2% wilt.


100.0
80.0
60.0
40.0
20.0
0.0
% Wilt
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days after inoculation
Rs-09-161
Rs-TssM-
Rs-TssH-
Rs-Hcp-
Rs-Omp-
Rs-Vgr-
Rs-hrcV

A

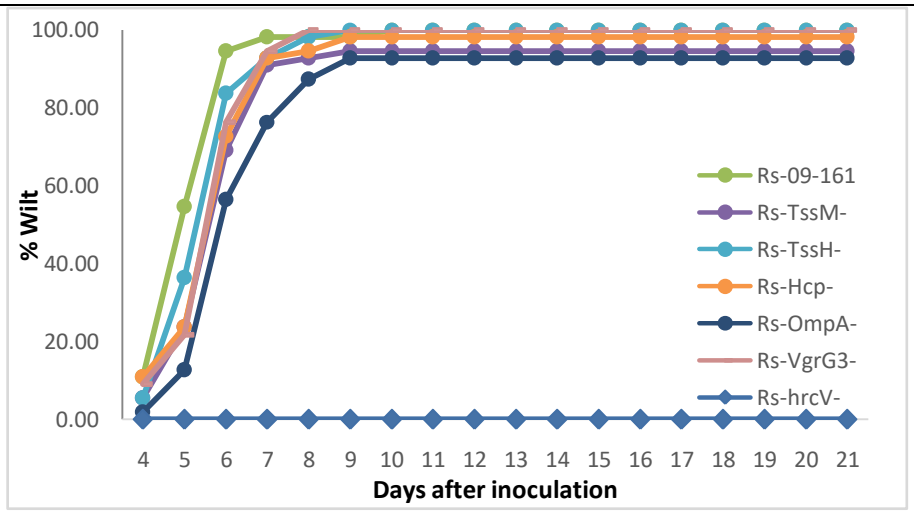

100.0
80.0
60.0
40.0
20.0
0.0
% Wilt
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days after inoculation
Rs-09-161
Rs-TssM-
Rs-TssH-
Rs-Hcp-
Rs-Omp-
Rs-Vgr-
Rs-hrcV

B


100
80
60
40
20
0
% Wilt
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days after inoculation
Rs-09-161
Rs-TssM-
Rs-TssH-
Rs-Hcp-
Rs-Omp-
Rs-Vgr-
Rs-hrcV

C

D



5.9. Bacterial wilt incidence in brinjal (cv., *Agassaim)* inoculated with *R. solanacearum* wild type strain Rs-09-161 and its T6SS mutants by cut petiole inoculation. (A) Experiment 1 conducted with 4 replications (B) Experiment 2 conducted with 4 replications (C) Experiment 3 conducted with three replications (D) Wilt % obtained by combining mean of all replications. The plants were observed for 21 DPI.

Table 5.8. Wilt incidence caused by the T6SS mutants in susceptible in brinjal (cv., *Agassaim)* by cut petiole inoculation.

DAI	Mean wilt %																	
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rs-09-161	10.9	54.5^{a}	94.5^{a}	98.2^{a}	98.2^{a}	98.2^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Rs-TssM^{-}	5.5	23.6^{bc}	69.1^{bc}	90.9^{a}	92.7^{ab}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}	94.5^{a}
Rs-TssH^{-}	5.5	36.4^{b}	83.6^{ab}	92.7^{a}	98.2^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Rs-Hcp^{-}	10.9	23.6^{bc}	72.7^{b}	92.7^{a}	94.5^{ab}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}	98.2^{a}
Rs-OmpA^{-}	1.8	12.7^{cd}	56.4^{c}	76.4^{b}	87.3^{b}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}
Rs-VgrG3^{-}	9.1	21.8^{bc}	76.4^{b}	94.5^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Rs-hrcV^{-}	0	0.0^{d}	0.0^{d}	0.0^{c}	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.00^{b}
CD (0.05)	NS	16.96	15.7	11.2	10.15	7.6	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3
CD (0.01)	NS	22.55	20.9	15	13.5	10.15	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7

Data obtained is a mean of 11 replications. Each replication consisted of 5 plants. The plants were observed for 21DPI. NS: Non significant

In a column, means followed by the same letter are not significantly different at 1% or 5%.

Moderately resistant variety-*Arka Rakshak*

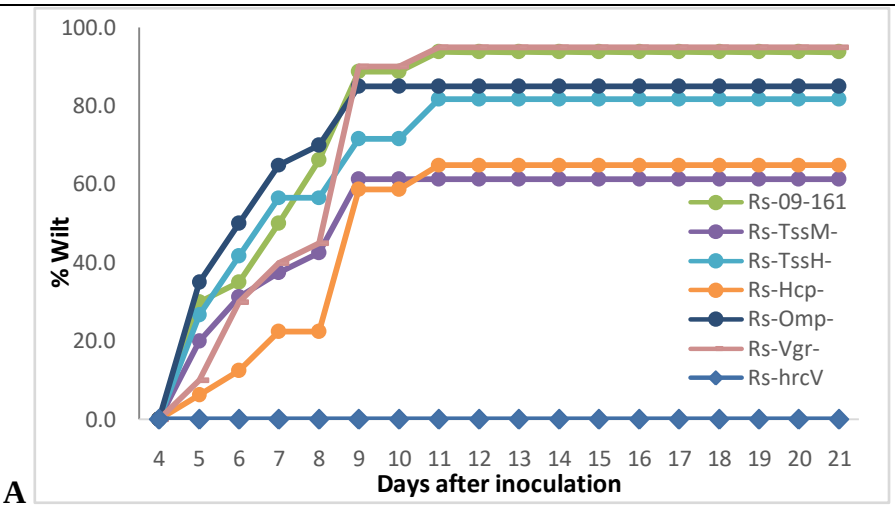
The virulence of *R. solanacearum* T6SS mutants on moderately resistant variety of tomato (*Arka Rakshak*) was tested in three individual experiments. In experiment 1, wilt incidence was observed on fifth day in wild type and the mutants. Wild type caused 30% wilt, whereas, in the T6SS mutant Rs-Hcp$^-$ and Rs-VgrG3$^-$ it was 6.3% and 10% respectively. Wild type caused 50% wilt on seven DAI whereas, in the T6SS mutants Rs-TssM$^-$, Rs-Hcp$^-$ and Rs-VgrG3$^-$ wilt incidence was less than 41%. A highest wilt of 94% was shown by wild type on 11 DAI whereas in the mutants Rs-TssM$^-$ and Rs-Hcp$^-$ it was less than 66% (Fig. 5.10a).

In experiment 2, wilt incidence was observed in wild type and majority of mutants on five DAI. Wild type caused more than 50% wilt on sixth DAI whereas in Rs-Hcp$^-$ and Rs-VgrG3$^-$ wilt incidence was less than 36%. Wild type caused a highest of 93% wilt on eighth DAI and the lowest was observed in Rs-VgrG3$^-$ (45%) followed by Rs-TssH$^-$ (70%) (Fig. 5.10b).

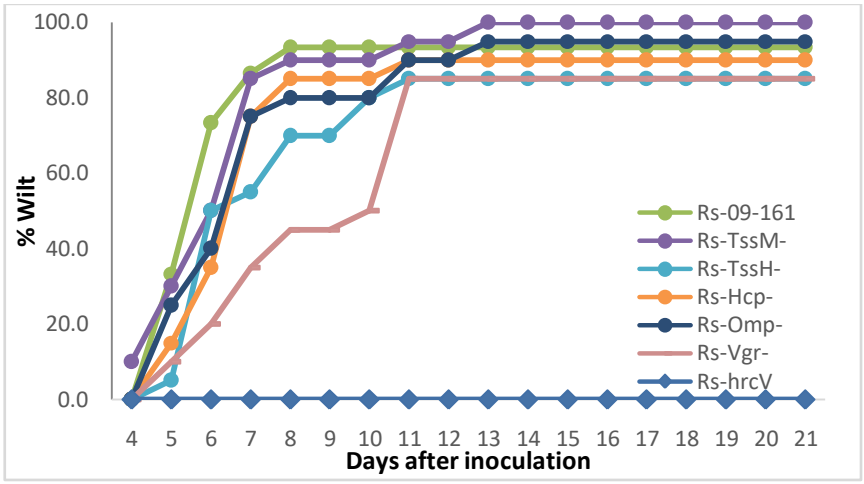
In experiment 3, wilt incidence was initiated on fourth DAI except for Rs-TssH$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$. Wild type caused more than 50% wilt on day five whereas in the T6SS mutants it ranged from 0-33%. Wild type caused 100% wilt 16 DAI and the lowest was observed in Rs-OmpA$^-$ (60%) and followed by Rs-TssM$^-$ (80%) (Fig. 5.10c).

The mean data of the experiments was obtained by combining the replications (Fig.5.10d and Table 5.9). Analysis of pooled data indicated that the mutants exhibited remarkable reduction in wilt incidence. Wilting of plants started 4 DAI except in Rs-TssH$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$. More than 50% wilt was observed in wild type on six DAI. In case of the mutants, wilting ranged from 22-39% on six DAI. Wild type caused a highest wilt 95.3%. Lowest wilt was displayed by Rs-Hcp$^-$ (80%), followed by Rs-TssM$^-$ (80.5%) and Rs-

OmpA$^-$ (81.8%). No wilt was observed in Rs-hrcV$^-$. On the seventh day, Rs-VgrG3$^-$ (36.3%), Rs-TssH$^-$ (44.2%) and Rs-Hcp$^-$ (46.3%) reduced the wilt incidence compared to the wild type (73.3%).

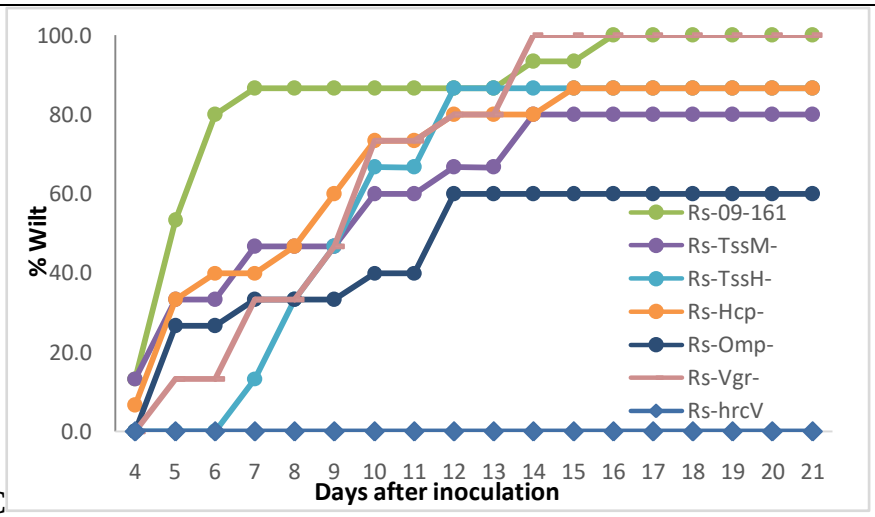


A

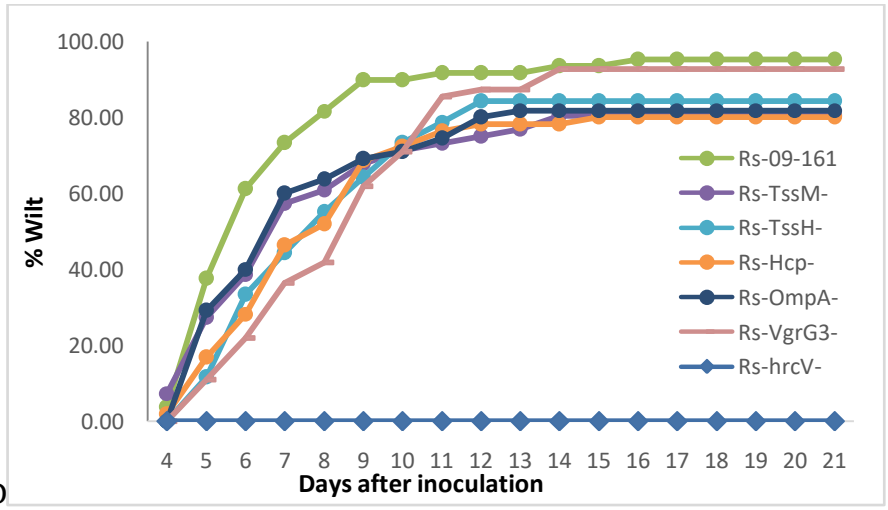


B

C



D



5.10. Bacterial wilt incidence in tomato (var., *Arka Rakshak)* inoculated with *R. solanacearum*wild type strain Rs-09-161 and its T6SS mutants by cut petiole inoculation. (A) Experiment 1 conducted with 4 replications (B) Experiment 2 conducted with 4 replications (C) Experiment 3 conducted with three replications (D) Wilt % obtained by combining mean of all replications. The plants were observed for 21 DPI.

Table 5.9.Wilt incidence caused by the T6SS mutants in moderately resistant variety of tomato(*Arka Rakshak*) by cut petiole inoculation.

DAI	Mean wilt %																	
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rs-09-161	3.6	37.5^{a}	61.2^{a}	73.3a	81.6^{a}	89.8^{a}	89.8^{a}	91.6^{a}	91.6^{a}	91.6^{a}	93.4^{a}	93.4^{a}	95.3^{a}	95.3^{a}	95.3^{a}	95.3^{a}	95.3^{a}	95.3^{a}
Rs-TssM^{-}	7.2	27.2^{ab}	38.6^{ab}	57.2ab	60.9^{ab}	67.7^{b}	71.3^{a}	73.1^{b}	75.0^{a}	76.8^{a}	80.4^{a}	80.4^{a}	80.4^{a}	80.4^{a}	80.4^{a}	80.4^{a}	80.4^{a}	80.4^{a}
Rs-TssH^{-}	0	11.5^{bc}	33.3^{b}	44.2b	55.1^{b}	64.2^{b}	73.3^{a}	78.7^{ab}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}	84.2^{a}
Rs-Hcp^{-}	1.8	16.8^{abc}	28.1^{b}	46.3b	51.8^{b}	68.6b	72.2^{a}	76.3^{ab}	78.1^{a}	78.1^{a}	78.1^{a}	80.0^{a}	80.0^{a}	80.0^{a}	80.0^{a}	80.0^{a}	80.0^{a}	80.0^{a}
Rs-OmpA^{-}	0	29.0^{ab}	40.0^{ab}	60.0ab	63.6^{ab}	69.0^{b}	70.9^{a}	74.5^{ab}	80.0^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}	81.8^{a}
Rs-VgrG3^{-}	0	10.9^{bc}	21.8^{bc}	36.3b	41.8^{b}	61.8^{b}	70.9^{a}	85.4^{ab}	87.2^{a}	87.2^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}	92.7^{a}
Rs-hrcV^{-}	0	0.0^{c}	0.0^{c}	0.0c	0.0^{c}	0.0^{c}	0.0^{b}	0.0^{c}	0.0^{b}	0.0^{b}	0.00^{b}	0.00^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}
CD (0.05)	NS	21.18	25.85	25.7	23.1	20.3	20.3	17.9	16.8	16.9	16.86	16.8	17	17	17	17	17	17
CD (0.01)	NS	NS	34.38	34.18	30.7	27.11	27.1	23.8	22.4	22.4	22.3	22.4	22.6	22.6	22.6	22.6	22.6	22.6

Data obtained is a mean of 11 replications. Each replication consisted of 5 plants. The plants were observed for 21 DPI. NS: Non significant

In a column, means followed by the same letter are not significantly different at 1% or 5%

The T6SS is involved in pathogenesis, bacterial interactions and competition (Silverman *et al.*, 2012). There is limited research available on the role of T6SS in virulence of *R. solanacearum*. The *in planta* virulence assay performed on *Arka Vikash* with Rs-09-161 and its T6SS mutants is depicted in Fig. 5.11. All the mutants delayed the wilt incidence on tomato and brinjal, when inoculated by drench and petiole method. Number of days taken to cause 50% and 75% wilt were compared (Table 5.10).

Fig. 5.11.*In planta* assays with *Arka Vikash* using *R. solanacearum* strains Rs-09-161 and its T6SS mutants: Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-VgrG3$^-$ and Rs-OmpA$^-$(left to right). The T3SS mutant Rs-hrcV$^-$ is used as a non-pathogenic (negative) control.

In *Arka Vikash*, two to three days delay was observed in Rs-TssH$^-$, Rs-Hcp$^-$ and Rs-VgrG3$^-$ to cause 75% wilt in the petiole inoculation. In the drench inoculation, delay of four to five days was observed in Rs-OmpA$^-$ to cause 75% wilt. In *Arka Rakshak*, Rs-VgrG3$^-$ caused 2-3 days delay to wilt 50% plants and 2-4 days delay was observed in all the mutants to cause 75% wilt.

In *Agassaim*, the T6SS mutants did not display any major delay in wilting in the petiole inoculation. However, prominent delay was observed in drench inoculation. Rs-TssM$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-TssH$^-$ delayed the wilt by 2-4 days and Rs-VgrG3$^-$ delayed the wilt by six days.

Table 5.10: Details about the number of days required to cause 50% and 75% wilt incidence in the inoculated seedlings

Crop	Variety	method of	Rs-09-161		Rs-TssM-		Rs-TssH-		Rs-Hcp-		Rs- OmpA-		Rs-VgrG3-		Rs-hrcV-	
		inoculation	50%	75%	50%	75%	50%	75%	50%	75%	50%	75%	50%	75%	50%	75%
Tomato	*Arka Vikash*	Petiole	5- 6	6-7	5-6	7-8	5-6	9-10	5-6	8-9	5-6	6-7	6-7	8-9	NA	NA
Tomato	*Arka Rakshak*	Petiole	5-6	7-8	6-7	12	6-7	10-11	6 -7	10-11	6-7	11-12	8-9	10-11	NA	NA
Brinjal	*Agassaim*	Petiole	4-5	5-6	5-6	6-7	5-6	5-6	5-6	6-7	5-6	7	5-6	6-7	NA	NA
Tomato	*Arka Vikash*	Soil drench	4-5	6-7	5-6	6-7	5-6	6-7	5-6	6-7	6 -7	11-12	5-6	6-7	NA	NA
Brinjal	*Agassaim*	Soil drench	6-7	8-9	8-9	12-13	9-10	12-13	8	11-12	8 -9	11-12	11-12	15	NA	NA

The number of days were calculated manually based on the mean data obtained after pooling all the replications.

The *vgrG* (valine-glycine repeat protein G) resemble bacteriophage tailspike and is secreted by the T6SS (Pukatzki *et al.,* 2009). *vgrG* is a trimeric protein which helps in assembly of the TssBC sheath and stacking of Hcp hexamers. It shares structural similarity with gp27/gp5 which forms the tail spike in T4. At the end of the *VgrG* protein, PAAR (Pro-Ala-Ala-Arg) repeat containing proteins bind to form a sharp tip of *HCp-VgrG* (Gallique *et al.,* 2017). The N-terminal region of *VgrG3* (VgrG3N) is an important component of T6SS apparatus and a C-terminal domain (VgrG3C) functions as a peptidoglycan-targeting glycoside hydrolase and is released as an effector in *Vibrio cholera* (Brooks *et al.,* 2013). The reduction in wilt observed by the Rs-VgrG3^{-} mutant could be associated with these functions of *VgrG* protein.

Rs-VgrG3- showed delay to cause 75% wilt in the petiole inoculated seedlings of tomato and drench inoculated seedlings of brinjal (Table 5.10). Based on the mean wilt data, Rs-VgrG3- has showed significant delay in wilt on petiole inoculated seedlings of tomato, *Arka Vikash* (Table 5.7), *Arka Rakshak* (Table 5.9) and drench inoculated seedlings of brinjal, *Agassaim* (Table 5.7) on day seven. The *vgrG* mutant strain of *Acinetobacter baumanni* caused reduced eukaryotic cell adherence and impaired lethality in mice (Wang *et al.,* 2018).

The *gene ompA* does not belong to the core components of the T6SS cluster but is associated with T6SS genes. Characterisation of T6SS gene components have revealed that *ompA, sciE* and *sciT* genes are found associated with only the virulent organisms (Shrivastava and Mande, 2008). It forms a translocation pore for translocation of type six effectors. *ompA* is present in all species of *E. coli, Yersinia, Pseudomonas, Acinetobacter, Burkholderia, Xanthomonas, Ralstonia, Hahella* and *Mesorhizobium* (Pautsch and Schulz, 1998). *OmpA* protein of *A. baumannii* is involved in pathogenesis by invasion of epithelial cells and death of dendritic

cells (Choi *et al.,* 2008; Lee *et al.,* 2010). *OmpA* forms a structural protein of the outer membrane which connects the outer membrane to the periplasmic peptidoglycan via its periplasmic domain. The attenuation in wilt caused by the Rs-OmpA$^-$ gene could be attributed to the role played by *OmpA* in virulent organisms. Except for petiole inoculated *Arka Vikash* seedlings, all other treatments have displayed delay in causing 75% wilt in the seedlings in comparison to the wild type strain Rs-09-161. Based on seventh day mean wilt data, Rs-OmpA$^-$ has showed significant delay in the drench inoculated seedlings of *Arka Vikash* (Table 5.5), *Agassaim* (Table 5.6) and petiole inoculated seedlings of *Agassaim* (Table 5.8). The delay in wilt is more prominent in case of the susceptible tomato and brinjal seedlings inoculated through the soil drench as compared to the petiole (Table 5.10).

Studies carried out by Zhang *et al* (2012), have demonstrated the importance of *tssM* gene in biofilm formation and virulence on tomato. The mutant Rs-TssM$^-$ has exhibited reduction in wilt in majority of the virulence assays. Colonization and proliferation studies on tomato with *tssM* deletion mutant in *R. solanacearum* have displayed significantly reduced bacterial cell numbers in the root and stem tissue (Zhang *et al.,* 2012). *TssM* forms an important component in the inner membrane of type six membrane complex which serves as a platform for assembling the inverted bacteriophage like secretion apparatus. Based on seventh day mean wilt data, Rs-OmpA$^-$ has showed significant delay in the drench inoculated seedlings of *Agassaim* (Table 5.6) and petiole inoculated seedlings of tomato, *Arka Vikash* (Table 5.7), *Arka Rakshak* (Table 5.9).

The gene *tssH* belongs to the AAA$^+$ superfamily of ATPases and a subfamily ClpV and plays an important role in assembly mechanism of the T6SS (Ramesh *et al.,* 2014b; Cascales and

Cambillau, 2012). The release of type six effectors through the cell membrane occurs by contraction of the tail sheath, TssH is involved in recycling of the contraction sheath (Gallique *et al.,* 2017). TssH could be playing a similar role in *R. solanacearum* and hence mutation of *tssH* gene has led to reduction in wilt incidence with Rs-TssH$^-$ mutant. Delay of 2-4 days was observed in petiole inoculated plants of tomato and drench inoculated plants of brinjal to cause 75% wilt as compared to wild type Rs-09-161 (Table 5.10). Based on seventh day mean wilt data, Rs-TssH$^-$ has showed delay in the drench inoculated seedlings of *Agassaim* (Table 5.6) and petiole inoculated seedlings of *Arka Rakshak* (Table 5.9).

The genes *hcp* (haemolysin co-regulated protein) resemble bacteriophage tail and issecreted as substrates along with *vgrG* by T6SS (Pukatzki *et al.,* 2009). *Hcp* is located on the inner tube of T6SS and is related to gp19 in phage T4 (Miyata *et al.,* 2013). Peng and co-workers (2016) identified three Hcp proteins in the whole genome of porcine extraintestinal pathogenic *E. coli* (ExPEC) with roles played in bacterial-eukaryotic cell interactions and bacterial competition. The attenuation in wilt displayed by Rs-Hcp$^-$ mutant could be probably attributed to one of the roles played by Hcp proteins. Rs-Hcp$^-$ caused a delay of 2-3 days to cause 75% wilt in petiole inoculated plants of tomato and drench inoculated plants of brinjal as compared to wild type Rs-09-161. Based on seventh day mean wilt data, Rs-Hcp$^-$ has showed delay in the petiole inoculated seedlings of *Arka Vikash* (Table 5.7) and *Arka Rakshak* (Table 5.9).

Many genes coding for the virulence factors like chemotaxis, flagella driven swimming motility, pili associated twitching motility, the extracellular polysaccharide (EPS), the Type Two Secretory System (T2SS) dependent cell wall degrading enzymes and the Type III

Secretory system (T3SS) have been studied (Schell, 2000; Saile *et al.,* 1997). The mutants of T2SS, chemotaxis, swimming motility and twitching motility display reduced virulence and mutants of EPS and T3SS are non-pathogenic (Saile *et al.,* 1997; Tans-Kersten *et al.* 2001; Meng *et al.,* 2011; Brito *et al.,* 2002). Our results are in agreement with those reported earlier for T3SS. In addition to this, the Type Six Secretion System (T6SS) has been recently identified in *R. solanacearum* (Zhang *et al.,* 2012), which is known to be involved in interbacterial interaction, biofilm formation and eukaryotic cell interaction. (Lossi *et al.,* 2012). Two genes of the T6SS: the *tssB* and *tssM* have been studied and reported to contribute towards the virulence of *R. solanacearum* in tomato (Zhang *et al.,* 2012; 2014). In this study, five different genes of the T6SS (including *tssM*) are been proved to be involved in virulence, the mutation of which has led to reduction of virulence in tomato and brinjal. The contribution of *tssH, hcp, ompA* and *vgrG3* in the virulence of *R. solanacearum* was not proved till date. This will prove as an important step in understanding the contribution of the individual genes of the T6SS in the virulence of *R. solanacearum*.

5.3.4. Inter-bacterial competition

The co-culture of T6SS mutants with model organism DH5α did not show any bacterial killing. Blue colonies of DH5α and white colonies of *R. solanacearum* mutants were visible on the co-cultured spots indicating no inter-bacterial killing. Similar results were observed in control plates where wild type was co-culturted with DH5α. The inter-bacterial competition assay is depicted in fig. 5.12.

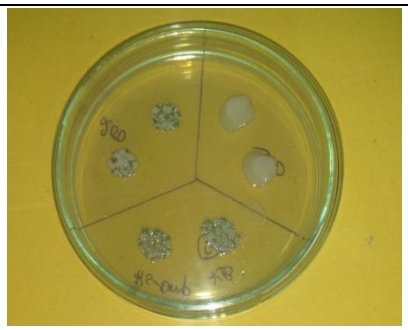


Fig.5.12. Interbacterial competition assay. Blue and white mixed colony patch represents co-culture of DH5α and *R. solanacearum*. Blue colonies are formed by DH5α and white fluidal colonies by *R. solanacearum*.

5.4. Conclusion

The mutants of T3SS and T6SS were tested for their virulence on brinjal and tomato seedlings by petiole inoculation and soil drench inoculation. The T3SS mutants; Rs-hrpB$^-$ and Rs-hrcV$^-$ were found to be non-pathogenic on susceptible brinjal and tomato in both methods of inoculation. These mutants can be used as an avirulent control in plant inoculation assays, in addition to their use in validating a putative T3E.

Five different mutants of the T6SS, Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$ were tested for virulence on susceptible tomato, brinjal and moderately resistant tomato. None of the mutants were found to be avirulent. However, it was observed that Rs-VgrG3$^-$ caused a delay of two to six days to cause 75% wilt in petiole inoculated seedlings of *Arka Rakshak* and drench inoculated seedlings of *Agassaim*. A delay of two to four days was observed by Rs-OmpA$^-$ in the petiole inoculated seedlings of *Arka Rakshak* and drench inoculated seedlings of *Agassaim* and *Arka Vikash* to cause 75% wilt. From these plant inoculation assays it is concluded that T6SS genes *viz.* (*vgrG3* and *ompA*) reduce the virulence (20-40%

compared to 57-98% by wild type) and delay the progress of wilt by two to four days. This study proves that T6SS genes of *R. solancearum* contribute towards the virulence in addition to other functions like bacterial interaction and competition.

CHAPTER VI

Validation of effector protein secretion through Type Three Secretion System

6.1. Introduction

The T3SS provides gram negative bacteria a unique virulence mechanism by which it can alter the biology of their eukaryotic host (Coburn *et al.,* 2007). The system works in a contact dependent manner and secretes proteins termed as T3Es from the cytosol of the bacterium into the cytoplasm of the eukaryotic host. This transfer of the effectors into the cytoplasm of the host is performed by a syringe like structure which pierces through the host cell. Both commensal and pathogenic bacteria express the T3SS, but, this system is more studied among the pathogenic bacteria as it plays a major role in their virulence (Puhar and Sensonetti, 2014). The T3SS is an emerging area of study among many molecular biologists working on plant and animal pathogens like *Pseudomonas, Xanthomonas, Salmonella* etc.

The T3SS is referred to as the main pathogenicity determinant of *R. solanacearum.* It is encoded by the hypersensitive response and pathogenicity (*hrp*) regulon in *R. solanacearum* (Boucher *et al.,* 1987). Any defect in the T3SS results in the loss of both the ability to induce hypersensitive responses and pathogenicity in plants (Alfano and Collmer, 2004). The T3SS is involved in the secretion of T3Es. Through bioinformatics analysis, 110 T3Es are identified across various isolates of *R. solanacearum*, with an average of 70-75 T3Es in individual isolate (Peeters *et al.,* 2013).

Many T3Es are validated in *R. solanacearum* through translocation studies using reporter based systems like the Cya reporter system and the HA reporter system (Cunnac *et al.,* 2004b; Mukaihara and Tamura, 2009; Mukaihara *et al.,*2010; Sole *et al.,* 2012). A protein can be validated as an effector protein secreted by the T3SS through translocation by detecting its presence in the wild type strain and its absence in its T3SS mutants. *hrpB* is the transcriptional regulator of T3SS and the T3Es (Genin *et al.,* 1992), and *hrcV* is a transmembrane component

of *hrp* pili, which is involved in translocation of the T3Es into the host cells. The *hrpB* mutant of *R. solanacearum* will fail to express the *hrpB*-dependent T3Es whereas the mutant of *hrcV* gene will fail to secrete the T3Es to the external environment.

For understanding how *R. solanacearum* manipulates plant cellular functions during infection, it is important to determine the complete repertoire of effector proteins possessed by it. There are many putative effectors identified through bioinformatics analysis and only a few validated through translocation or secretion analysis. Seventy two T3Es were identified in *R. solanacearum* isolate Rs-09-161 and 77 T3Es in Rs-10-244 through bioinfomatic analysis (Asolkar and Ramesh, 2018a). The details of these effectors are described in chapter III. There is a need to validate all the T3Es of *R. solanacearum*. *R. solanacearum* isolateRs-09-161 was selected as a representative isolate to validate two T3Es and the following objective was proposed "Validation of effector protein secretion through Type Three Secretion System".

6.2. Materials and Methods

6.2.1. Selection of gene

Two T3E genes RipAM and Rs_T3E_Hyp15 ('RS15E' hereafter) identified in *R. solanacearum* isolate Rs-09-161 through bioinformatics analysis (Asolkar and Ramesh, 2018a) were selected as putative T3Es for validation through secretion studies. These genes were selected based on the literature available on validated T3Es of *R. solanacearum*. The two selected T3E genes were not validated as T3E at the time of initiation of this work. The sequences of the genes were extracted from the annotated data of Rs-09-161 (Asolkar and

Ramesh, 2018a). Details of the genes are given in the table 6.1. The details of plasmids and the strains used in this study are given in table 6.2.

Table 6.1. List of genes used in the study, details about the locus tag, size and location in Rs-09-161.

Gene	Locus tag in Rs-09-161	Locus tag in standard strain	Location	Size of the gene (bp)
RipAM	161_30700	RSc3272	Chromosome	465bp
Rs_T3E_Hyp15 ('RS15E')	161_35100	RSPsi07_1860	Megaplasmid	2799

RSc/RSp: GMI1000, RPSI07: Psi07

6.2.1.1. Amplification of T3E gene:

The primers were designed for the amplification of genes as mentioned in pENTR Directional TOPO cloning Kit (Thermo Fisher Scientific, USA). pENTR Directional TOPO cloning kit was used for the directional cloning of blunt end PCR products into an entry vector and subsequent transfer to the GATEWAY destination vectors.

The forward primer was designed with the start codon (ATG) in frame and with a CACC overhang at the 5'- end. The reverse primer was designed from the codon just upstream of the stop codon. Since the gene will be amplified without the stop codon, it can be fused in frame with a C-terminal tag after the recombination of entry clone with the pRCG-Pep-GWY destination vector. The sequences of the primer are given in table 6.3. The gene sequences were amplified using a Hot-start PCR with blunt end proof reading Phusion high fidelity DNA polymerase (Thermo Fisher Scientific, USA). The amplifications were performed in Mastercycler Pro (Eppendorf, GmBH) and the amplicons were visualised on 1.0% agarose gel

Table 6.2.List of strains and plasmids used in this study.

Strains/ Plasmids	Relevant genotype	Source/Reference
Plasmids		
pENTR-SD-D-TOPO	entry vector used for the cloning of T3E genes	Thermo Fisher Scientific, USA
RipAMpENTR-SD-D-TOPO	RipAM gene cloned in pENTR-SD-D-TOPO entry vector	This study
RS15EpENTR-SD-D-TOPO	RS15E gene cloned in pENTR-SD-D-TOPO entry vector	This study
pRCG-Pep-GWY	Destination vector with HA sequence tag.	Monteiro *et al.,* 2012a
pRCG-Pep-RipAM	RipAM gene cloned in pRCG-Pep destination vector	This study
pRCG-Pep-RS15E	RS15E gene cloned in pRCG-Pep destination vector	This study
E. coli strains		
S17-1λpir	KmR, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir.	Plant Pathology Laboratory
Ec-RipAM	RipAM::pENTR-SD-D-TOPO	This study
Ec-RS15E	RS15E::pENTR-SD-D-TOPO	This study
One Shot® ccdB Survival™ 2 T1R chemically competent cells	ccdB resistant cell for maintanance of pRCG-Pep-GWY vector	
Ec-pRCG-Pep-GWY	pRCG-Pep-GWY	This study
Ec-pRCG-RipAM	pRCG-Pep-RipAM	This study
Ec-pRCG-RS15E	pRCG-Pep-RS15E	This study
R. solanacearum strains		
Rs-RipAM	pRCG-Pep-RipAM in wild type strain Rs-09-161	This study
Rs-hrpB-RipAM	pRCG-Pep-RipAM cloned in Rs-hrpB- mutant	This study
Rs-hrcV-RipAM	pRCG-Pep-RipAM cloned in Rs-hrcV- mutant	This study
Rs-RS15E	pRCG-Pep-RS15E wild type strain Rs-09-161	This study
Rs-hrpB-RS15E	pRCG-Pep-RS15E cloned in Rs-hrpB- mutant	This study
Rs-hrcV-RS15E	pRCG-Pep-RS15E cloned in Rs-hrcV- mutant	This study

containing 0.5 μg.mL^{-1} of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The amplified products were purified using GeneJETTM PCR Purification Kit (Fermentas Life Sciences, EU) as per manufacturer's instructions and quantified using Nano drop-1000, Thermo fisher scientific, USA. The PCR reaction mixture and standardizd conditions are given in table 6.4.

Table 6.3. List of primers used for the amplification of T3E genes

Gene	Primer	Primer sequence (5'-3')	Amplicon (bp)
RipAM	RipAMF	CACCATGAAACGAATCGACGTCCAC	462
	RipAMR	GGCCGGCCGCGTCGTTGT	
RS15E	RS15F	CACCATGCCGATTAGTGCTTGC	2796
	RS15R	TGATTGATGTATCAAAGACAGCGT	

Table 6.4. The reaction mixture and PCR conditions for amplification of T3E gene

Gene	Reaction mixture	PCR conditions
RipAM	20 μL reaction mixture contained 0.3 μM of each primer, 200 μM dNTPs, 1 X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 50 ng of the DNA template check gel photos	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 sec, 70°C for 30sec, 72°C for 30 s and final extension of 10min at 72°C.
RS15E	20 μL reaction mixture contained 0.5 μM of each primer, 200 μM dNTPs, 1X assay buffer, 0.5U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 sec, 62°C for 30sec, 72°C for 3 min and final extension of 10 min at 72°C.

6.2.2. Cloning in pENTR-SD-D-TOPO entry vector:

The amplified genes *RipAM* and *RS15E* were cloned in pENTR-SD-D-TOPO vector supplied with pENTR Directional TOPO cloning Kit. The pENTR-SD-D-TOPO vector is a modified entry vector and has the presence of Topoisomerase I bound at both the 3'-ends

and a GTGG overhang at the 5'-end of the cloning site downstream of the promoter. The cloning site of the vector is flanked with attL regions which recombines with attR regions present on the GATEWAY destination vector and transfers the cloned gene to the destination vector. This vector has the presence of ribosome binding site (RBS) and a T7 gene 10 translational enhancer for optimal expression of native protein after recombination with a Gateway® destination vector. Optimum molar ratio of 1:1 was maintained for PCR product: entry vector as mentioned by the manufacturer. Ligation was carried out as follows:

Table 6.5. Ligation reaction for cloning of effector gene in pENTR-SD-D-TOPO vector

	Volume added(µl)		
Reactants	*RipAM*	*Rs-15E*	Control reaction
Amplified product	2.0	2.0	2.0
Salt solution	1.0	1.0	1.0
Sterile H2O	2.0	2.0	2.0
pENTR-SD-D-TOPO vector	1.0	1.0	1.0

The ligation reaction was incubated at 22ºC for 30 min and then placed on ice until transformation in *E. coli*. Control reaction was also carried out under same condition. The vector map of the pENTR-SD-D-TOPO is given in Fig 6.1.

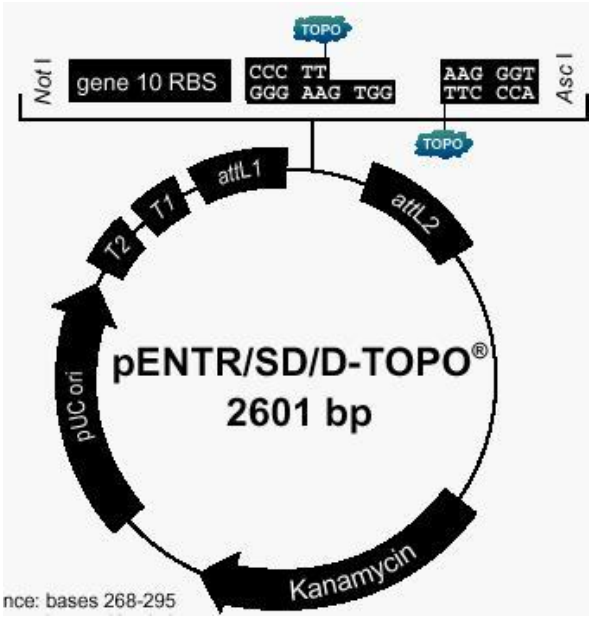


Fig. 6.1.Vector map of pENTR-SD-D-TOPO entry vector. The vector has the presence of Topoisomerase I at the 3'-ends and GTGG overhang at the 5'-end of the cloning site which helps in correct orientation of the insert gene. The gene cloned in this vector is preceeded by the RBS which is transferred to the destination vector during LR recombination and helps in the optimal expression of the native proteins.

6.2.2.1. Transformation:

Transformation was carried out using One Shot TOP10 competent *E. coli* cells supplied along with the entry vector kit as per manufacturer's instructions. One vial of TOP10 cells (50 µL) were mixed gently with two µL of ligation reaction mix for *RipAM*, *Rs_T3E_Hyp15* and control reaction each respectively. The cells were maintained on ice for 30 min and then proceeded for transformation. Transformation was carried out by heat shock method at 42ºC for 30 s. Immediately after the heat shock, the cells were transferred to ice cold conditions and 250 µl of SOC medium was added. The vials were then incubated at 37ºC for 1h at 200 rpm. After 1h, the transformed cells were spread plated on pre-warmed LB-Kanamycin (50 $\mu g.mL^{-1}$) plates and incubated overnight at 37ºC.

6.2.2.2. Analysis of the recombinants:

The positive clones were transferred to LB plates containing kanamycin (50 µg.mL^{-1}) and incubated at 37°C. After 24 h, the orientation of the gene in the entry vector was confirmed by colony PCR.

6.2.2.2.1. Colony PCR:

Colony PCR was carried out with M13 reverse primer and specifically designed diagnostic primer in the internal region of *RipAM* and *RS15E* respectively. The sequences of the primers are given in the table 6.6. The *E.coli* colonies were used as a template instead of DNA for PCR. Amplification of gene with internal primer and M13 primer will confirm the orientation of the T3E gene in the entry vector with respect to ribosome binding site. The amplifications were performed in in Mastercycler Pro (Eppendorf, GmBH) and documented using MultiImage Light Cabinet (Alpha Innotech Corporation). The amplicons were visualised on 1.0% agarose gel containing 0.5 µg.mL^{-1} of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The PCR reaction mix and standardized conditions are given in table 6.7.

Table 6.6. Sequence of primers used for the confirmation of T3E gene in recombinant entry vector

Gene	Primer	Primer sequence (5'-3')	Amplicon (bp)
RipAM	RipAMD	ATGGACGTGCTCAGCAAC	338
	M13R	CAGGAAACAGCTATGACC	
RS15E	RS15D	CGTGCGGAACAACAAGGG	506
	M13R	CAGGAAACAGCTATGACC	

Table 6.7. Reaction mixture and PCR conditions for confirmation of T3E gene in recombinant entry vector.

Gene	Reaction mixture	PCR conditions
RipAM	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 0.5 U Taq DNA polymerase and 1 recombined *E.coli* colony	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 64°C for 30s, 72°C for 30 s and final extension of 10min at 72°C.
RS15E	20 µL reaction mixture contained 1 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 0.5 U Taq DNA polymerase and 1 recombined *E.coli* colony	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 30 s and final extension of 10min at 72°C.

6.2.2.2.2. Restriction digestion:

The transformed *E.coli* colonies confirmed with colony PCR were grown in LB broth containing 50 $\mu g.mL^{-1}$ kanamycin overnight at 37°C under shaker condition of 150 rpm. The plasmid was isolated using plasmid isolation kit (GeneJET Plasmid Miniprep Kit, #K0502) as per the manufacturer's instructions. The eluted plasmid was quantified using Nano drop-1000, Thermo fisher scientific, USA and used for restriction digestion for confirmation of the recombination. To obtain the restriction profile, the sequence of the T3E gene without the stop codon was inserted in the cloning site of the pENTR-SD-D-TOPO entry vector. This recombined sequence was then submitted to NEB Cutter 2.0 and analysed as a circular plasmid. Specific restriction enzymes were selected through custom digest and the fragment sizes obtained were correlated with the gel photographs after visualisation. In the reaction mixture, 5 units of each restriction enzyme (Thermo Scientific, USA) and approximately 200 ng of recombined plasmid was used. Restriction digestion was carried out at 37°C for 1h; the fragments were separated on 1.2% agarose

gel and documented. Detailed procedure for restriction digestion is described in section 4.2.3.3.2. The restriction profile of the recombined vector is given in table 6.8. The confirmed colonies were maintained as 30% glycerol stock at -80°C.

Table 6.8.Restriction profile of the recombined entry vectors.

Sr. no.	Recombined plasmid	Restriction enzyme	Band size (bp)
1	RipAMpENTR-SD-D-TOPO	*Apa* I	3063
		Eco RV/*Pst* I double digest	2605, 458
		Sac II	2595, 468
		SaC I/ *Not* I	2707, 356
2	RS15EpENTR-SD-D-TOPO	*Not* I linearisation	5397
		Eco RI/ *Eco* RV double digest	4204, 1193
		Apa I/ *Dra* I	4641, 756

6.2.3. pRCG-Pep-GWY destination vector:

The pRCG-Pep-GWY vector was obtained from Dr. Marc Valls (Monteiro *et al.*, 2012b) in the form of dry pellet. The pellet was dissolved in 10 µL of sterile MilliQ water and used for transformation with One Shot® *ccdB* Survival™ 2 T1R chemically competent cells (Thermo Fisher Scientific, USA). Transformation was performed using heat shock method at 42ºC for 30 s using 100 ng of plasmid DNA as per manufacturer's recommendation.

The positive clones were selected on LB agar containing ampicillin (50 µg.mL^{-1}) and gentamycin (50 µg.mL^{-1}). The selected clones were inoculated in LB broth with the desired antibiotics and grown under shaker conditions of 150 rpm overnight using GeneJET Plasmid Miniprep kit (#K0502, Thermo Scientific, USA) as per manufacturers instructions. The pRCG-Pep-GWY plasmid was isolated from the positive colony and confirmed by restriction digestion. The sequence of pRCG-Pep-GWY was analysed using

NEB Cutter 2.0 for selection of specific restriction enzymes. The restriction profile obtained after restriction digestion was compared with that of NEB Cutter fragments and confirmed.

The circular vector has the presence of two regions of homology to the *R. solanacearum* chromosome (GMI1000) which constitutes a pseudogene. These two regions are present flanking the integration element. The integration element is made up of gentamycin resistance gene (also confers resistance to apramycin sulphate) and the GATEWAY cassette. The gene of interest with an open reading frame and without the stop codon is cloned into the vector by replacing the GATEWAY cassette. Downstream of the attR2 region is present a hemagglutinin (HA) epitope tag sequence. Since the T3E gene is cloned without the stop codon, it should produce HA epitope tagged proteins. Both the ends of the integration element have the presence of transcriptional terminators (*Tfd* terminator and *T7* terminator). The genes cloned in the pRCG-Pep-GWY vector are under the control of *Pep* promoter which regulates the synthesis of *eps* operon. The *Pep* promoter is very active under high cell densities *of R. solanacearum* and is located upstream of the attR site(Monteiro *et al.,* 2012b). The GATEWAY cassette is made up of the *ccdB* gene, the product of which acts as an inhibitor for DNA gyrase and leads to the death of the bacteria (Bahassi *et al.,* 1999). The pRCG-Pep-GWY vector is therefore maintained in *ccdB* resistant cells. The vector map of pRCG-Pep-GWY vector is given in fig. 6.2.

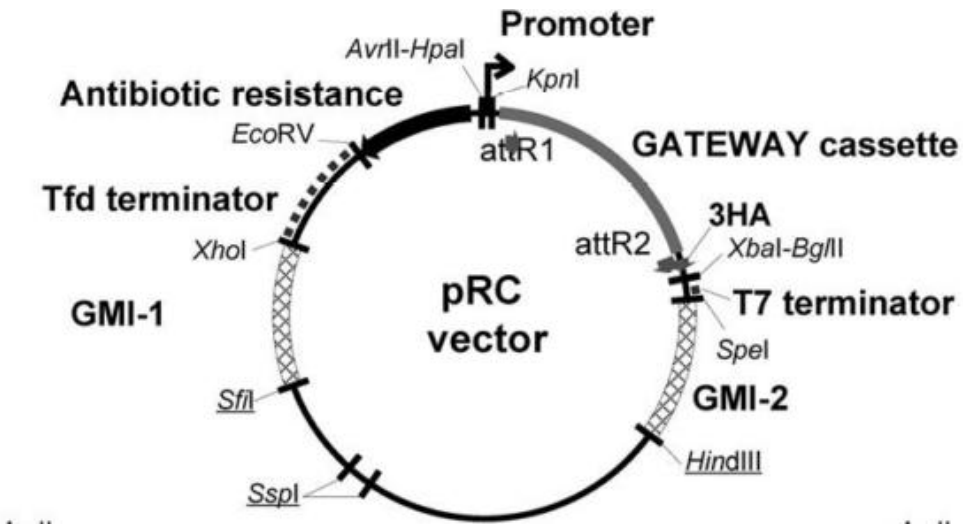


Fig. 6.2.Backbone of pRCG vector series. The pRCG-Pep-GWY vector has the presence of Pep (*eps)* promoter and gentamycin resistant gene in the antibiotic resistance cassette. The vector gets integrated into the chromosome of *R. solanacearum* through the GMI-1 and GMI-2 region. The GATEWAY cassette is present within the attR regions and is replaced by the T3E. The cloned T3E is secreted as a HA fused protein. The HA tag sequence is located immediately downstream of the attR2 region.

6.2.4. LR recombination reaction:

The effector genes RipAM and RS15E were transferred from pENTR-SD-D-TOPO entry vector to the pRCG-Pep-GWY destination vector by LR clonase reaction. LR recombination reaction was set up with Gateway® LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, USA) as per manufacturer's instructions. The LR reaction mix consisted of 150 ng of entry vector with the T3E, 150 ng of PRCG-Pep-GWY destination vector and two microlitre of LR Clonase reaction mix in a ten µL reaction system. The tubes were maintained at 25ºC for 1 h. For termination of reaction, 1 µL of Proteinase K was added and the tubes were incubated at 37ºC for 10 min. LR recombination reaction catalyzes *in vitro* transfer of DNA segments present between attL region of entry vector to attR region of destination vector, thereby creating an attB expression clone. This resulted in the

transfer of effector genes from RipAMpENTR-SD-D-TOPO and RS15EpENTR-SD-D-TOPO to pRCG-Pep-RipAM and pRCG-Pep-RS15E respectively. The resultant effector construct present in the LR reaction was transferred to *E.coli*. pENTR-gus vector was used as an entry vector in the control reaction.

6.2.4.1. Electroporation:

The pRCG-Pep-Effector constructs present in the LR mix were transferred to *E. coli* S17-1λpir strain by electroporation. *E.coli* S17-1λpir was inoculated in LB broth to an initial OD_{600nm}=0.1 and allowed to grow under shaking conditions at 160 rpm. The actively growing cells were harvested at 0.5 OD and electrocompetent cells were prepared. Detailed procedure for preparation of electrocompetent cells was given in section 4.2.4.1. Briefly, electrocompetent cells were prepared by serial washing of equal volume ice cold water, half volume ice cold water, one fourth volume 10% ice cold glycerol and finally in /100^{th} volume of 10% glycerol at 8000 rpm for 10 min at 4°C (Asolkar and Ramesh, 2018b).

Electroporation was performed in 0.1 cm cuvettes at 1.5 kV with 100 µL of cells mixed with 4 µL of LR reaction mix in micropulser (Bio-Rad). The cells were immediately re-suspended in 700 µL of LB broth and were maintained under shaking conditions of 200 rpm for 2 h at 37ºC. The cells were pelleted at 4000 rpm for 3 min and re-suspended in 100 µL of LB broth. The suspended cells were plated on BG agar plates containing ampicillin (50 $\mu g.mL^{-1}$) and apramycin (50 $\mu g.mL^{-1}$), and incubated overnight at 37ºC. The colonies obtained were used for confirmation of presence of effector constructs by colony PCR. The primers and PCR conditions were same as mentioned for amplification of the RipAM and RS15E respectively (Table 6.3, 6.4). The recombined *E.coli* cells were used as a template instead of template DNA. The amplifications were performed in

Mastercycler Pro (Eppendorf, GmBH) and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The resultant effector construct pRCG-Pep-RipAM and pRCG-Pep-RS15E was isolated from Ec-pRCG-RipAM and Ec-pRCG-RS15E and transferred to wild type *R. solanacearum*.

6.2.5. Transfer of effector constructs to *R. solanacearum*

6.2.5.1. Transfer of effector constructs in Rs-09-161

The pRCG-Pep-RipAM and pRCG-Pep-RS15E effector constructs were transferred to wild type *R. solanacearum* strain Rs-09-161 by electroporation. Detailed procedure for electroporation is given in section 4.2.4.1.

Electroporation was performed in 0.1cmcuvettes at 2 kV with 100 µL of cells mixed with 2 µg of pRCG-Pep-RipAM and pRCG-Pep-RS15E effector constructs respectively in micropulser (Bio-Rad). The cells were immediately re-suspended in 700 µL of BG broth and were maintained under shaker conditions of 200 rpm for 2 h at RT. The cells were pelleted at 4000 rpm for 3 min and resuspended in 100 µL of BG broth. The suspended cells were plated on BG agar plates containing ampicillin (50 $\mu g.mL^{-1}$) and apramycin (50 $\mu g.mL^{-1}$), and incubated for 48-72 h. The colonies obtained were used for confirmation of presence of effector constructs.

6.2.5.1.1. Confirmation of effector constructs in Rs-09-161

Positive colonies obtained in the presence of antibiotics were confirmed for the presence of pRCG-Pep-RipAM and pRCG-Pep-RS15E effector constructs using specifically designed internal primers and HA tag reverse primer. The extraction of genomic DNA was carried out using procedure as described by Ramesh *et al.* (2014a) and was described in

section 4.2.2.2. The sequences of the primers and standardised PCR conditions are given in table 6.9 and 6.10. All the amplifications were performed in Mastercycler Pro (Eppendorf, GmBH). The amplicons were visualised on 1.0% agarose gel containing 0.5 $\mu g.mL^{-1}$ of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). Rs-RipAM and Rs-RS15E were then maintained as 30% glycerol stock at -80°C and routinely grown in the presence of ampicillin (50 $\mu g.mL^{-1}$) and apramycin(50 $\mu g.mL^{-1}$).

Table 6.9. Sequences of primers used in the confirmation of effector construct in *R. solanacearum.*

Gene	Primer	Primer sequence (5'-3')	Amplicon (bp)
RipAM	RipAMF	CACCATGAAACGAATCGACGTCCAC	675
	HAR	CCCGCATAGTCAGGAACATCG	
RS15E	RS15F	CACCATGCCGATTAGTGCTTGC	3009
	HAR	CCCGCATAGTCAGGAACATCG	

Table 6.10. The reaction mixture and PCR conditions for confirmation of effector construct in *R. solanacearum*.

Gene	Reaction mixture	PCR conditions
RipAM	20 µL reaction mixture contained 1.0 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 3% DMSO, 3 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 45 s and final extension of 10 min at 72°C.
RS15E	20 µL reaction mixture contained 1.0 µM of each primer, 200 µM dNTPs, 1 X assay buffer, 0.5 U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min and final extension of 10 min at 72°C.

6.2.5.2. Transfer of effector constructs to T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$.

The pRCG-Pep-RipAM and pRCG-Pep-RS15E effector constructs were transferred to Rs-hrpB$^-$ and Rs-hrcV$^-$ through natural transformation. The genomic DNA of Rs-RipAM and Rs-RS15E was used as donor DNA in respective natural transformation.

Natural transformation was carried out as mentioned in Boucher *et al.* (1985) with slight modifications. A single colony of recipient cells Rs-hrpB$^-$ and Rs-hrcV$^-$ was grown in one fourth strength of M63 medium (Appendix II) supplemented with 1M $MgSO_4$, 0.2% glucose, and 50 µg.mL^{-1} ampicillin. The cells were grown to a population of 5x10^8 CFU.mL^{-1} for natural transformation, 100 µL of cells were mixed with 2 µg of donor genomic DNA and maintained undisturbed for 2 min. The mixture was then patched in triplicates on one fourth strength of M63 agar (supplemented with $MgSO_4$, and glucose) without antibiotic and incubated at room temperature for 24 h. After 24 h, the patches were mixed thoroughly with loop and streaked on BG agar containing ampicillin (50 µg.mL^{-1}) and apramycin (50 µg.mL^{-1}). The plates were incubated at RT for 48 h and observed for well-formed colonies. The colonies obtained were transferred to BG broth with required antibiotics for isolation of genomic DNA. Confirmation of pRCG-Pep-RipAM and pRCG-Pep-RS15E was carried as described in section 6.2.5.1.1. The confirmed Rs-hrpB-RipAM, Rs-hrpB-RS15E, Rs-hrcV-RipAM and Rs-hrcV-RS15E were maintained as 30% glycerol stock and routinely cultured in the presence of ampicillin (50 µg.mL^{-1}) and apramycin(50 µg.mL^{-1}).

6.2.6. Precipitation of protein

Since the effectors are cloned without the stop codon in pRCG-Pep-RipAM and pRCG-Pep-RS15E vector, they will be secreted as HA epitope tag fused proteins. These proteins

can be detected in the cell supernatants as well as in the bacterial cells. The proteins from the bacterial cells were obtained by cell lysis and from the supernatant by TCA-Acetone precipitation (Monteiro *et al.*, 2012a).

A single colony of Rs-RipAM, Rs-RS15E and its T3SS mutant expressing effector constructs Rs-hrpB-RipAM, Rs-hrpB-RS15E, Rs-hrcV-RpAM and Rs-hrcV-RS15E was grown in BG broth containing ampicillin (50 µg.mL^{-1}) and apramycin(50 µg.mL^{-1}) under shaker conditions of 150 rpm at RT for 16-18 h respectively. These cultures were transferred to 40 mL of M63 medium (supplemented with 1 M $MgSO_4$, 0.2% glucose, 100 µg.mL^{-1} congo red, 50 µg.mL^{-1} ampicillin and 50 µg.mL^{-1} apramycin) at an initial density of OD_{600nm} = 0.1. The flasks were grown till exponential phase under shaker conditions of 150 rpm at RT for 16 h. The cultures were pelleted by centrifugation at 8000 rpm for 10 min at RT. The culture supernatant was separated and proteins were precipitated using one-fourth volume of 25% Trichloroacetic acid. The tubes were incubated overnight at 4°C.

The cell pellet obtained was re-suspended in 500 µL of 1 X Phosphate buffered saline (PBS) buffer and lysed by sonication using Vibra-cell sonicator (Sonics, USA) with an amplitude of 50% for 1 min 30 s. The pulse was passed for 20 s thrice with 10 s break in between wherein the tubes were placed on ice. The lysate was centrifuged at 5000 rpm for 5 min for the removal of cell debris. The supernatant was transferred to fresh eppendorf tubes and maintained at -80°C until use.

The proteins precipitated by TCA precipitation were pelleted by centrifugation at 8000 g for 30 min at 4°C. The pellets were washed in 20 mL of cold 90% acetone and centrifuged at 8000 g for 30 min at 4°C. The washing step was repeated again with 10 mL of cold 90% acetone. The pellets were air dried, re-suspended in 100 µL of PBS buffer and maintained

at -80°C until use. The cell lysates and the precipitated proteins were used for immuno detection.

6.2.6.1. Quantification of proteins

The precipitated proteins were quantified by the Bradford's protein assay (Bradford, 1976). Twenty µL of protein sample was mixed with one mL of 1X Bradford's dye and incubated at room temperature for 5 min. The absorbance was read at 595 nm using Nanodrop 1000 (Thermo Scientific, USA). Protein concentration in the samples was determined using standard curve extrapolated using BSA in the liner range of 0.1 -1.0 $mg.mL^{-1}$

6.2.7. SDS PAGE

SDS PAGE was performed as described by Sambrook and Russell (2001). SDS PAGE was performed in vertical gel apparatus (Atto Corporation, Japan) as per manufacturer's instructions. The plates were assembled and sealed from three sides with the help of gasket provided by the manufacturer. PAGE gel was casted using 6% of stacking gel and 10% of resolving gel without the formation of air bubbles. After polymerization, the gel plate assembly was placed into the electrophoresis unit filled with 1 X tris-glycine buffer. Thirty microliters samples from cell lysate as well as protein precipitate were mixed with ten microliters 6 X Laemmli buffer, maintained at 75°C water bath for five min and loaded in the respective wells. Color bust marker, molecular weight 8000 Da to 22000 Da (Sigma Aldrich) was used for reference. Electrophoresis was carried with a constant current of 45 mA and a voltage of 150 V for 2 h.

6.2.7.1. Electrotransfer

The proteins separated during PAGE were transferred to precut 0.2 µm polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, USA) as per manufacturer's instructions. The membrane was pre-wet in 100% ethanol for 5 min and drained. The membrane was then equilibrated by soaking it in the transfer buffer. Filter paper was also saturated in transfer buffer (Appendix III) for 5 min. The sandwich 'blot' was prepared on the transfer apparatus on the lower electrode (anode). The base layer consisted of four layers of filter paper, followed by the membrane, the gel and finally four additional filter papers. The air gap present between the layers was removed by gently rolling a glass rod from the center of the sandwich towards the edges. Electrotransfer was performed using semidry electro transfer unit (Atto Corporation, Japan) at 120 mA for 70 min. After electroblotting, membrane was washed twice with 20 mL of sterile MilliQ water for 5 min to remove the gel and transfer buffer components. The membrane was used for immunodetection by western blotting.

6.2.8. Western Blotting

Western blotting was performed using Western Breeze® chromogenic immunodetection kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. All the steps were carried out under shaker condition of 50 rpm at RT in a covered plastic petridish. The nonspecific background binding of antibodies on the membrane was blocked by incubating the membrane in 10 mL of blocking solution for 30 min. The membrane was rinsed with 20 mL of sterile MilliQ water twice for 5 minutes each. The details of the antibodies used are given in table 6.11.

Table 6.11. Primary and secondary antibody used in Western blotting.

Name of the antibody	Purpose	Working concentration
Rabbit anti-HA polyclonal antibody	Primary antibody against HA epitope tag	0.2 $\mu g.mL^{-1}$
Alkaline phosphatase conjugated anti-rabbit goat antibody	Anti-rabbit secondary antibody	Pre-diluted to working stock

Rabbit anti-HA polyclonal antibody (Thermo Fisher Scientific, USA) was diluted to a working stock of 0.2 $\mu g.mL^{-1}$ in 10 mL of primary antibody solution provided by the manufacturer. The membrane was incubated in primary antibody solution overnight at 4°C under stationary condition. Following this, the membrane was washed with 20 mL of 1 X antibody wash solution for 5 min. The washing step was repeated 3 times to remove residual unbound primary antibody. Ten mililitres of secondary antibody solution (supplied in the kit) was added to the membrane and incubated for 30 min. Alkaline phosphatase conjugated anti-rabbit goat antibody was used as the secondary antibody. The membrane was washed with 20 mL of 1 X antibody wash solution for 5 min. the washing step was repeated three times to remove any unbound or nonspecific bound secondary antibody. The membrane was rinsed with 20mL of sterile MilliQ water twice for three min each.

The membrane was incubated with 5 mL of chromogenic substrate solution for 60 min for chromogenic detection. A solution of 5-Bromo-4-Chloro-3-indolyl-1-Phosphate (BCIP) and nitro blue tetrazolium (NBT) was used as the substrate for alkaline phosphatase. After colour development (Purple colored bands), the membrane was rinsed with 20 mL of MilliQ water twice for two min each. The membrane was air dried and photographed.

6.2.9. RNA isolation

RNA isolation was carried out using aseptic RNA handling technique. The surfaces of the working table and instruments were cleaned with RNase inhibitor (Thermo Fisher Scientific, USA). The plastic ware used in RNA isolation was soaked in 0.1% DEPC solution overnight, air dried and sterilized by autoclaving.

A single colony of Rs-RipAM and Rs-hrpB-RipAM was grown in 10 mL of one fourth strength M63 broth for 16-18 h under shaker condition of 150 rpm. One mL of bacterial suspension of density 2 x 10^8 CFU.mL^{-1} was centrifuged at 8000 rpm for 10 min at RT. The supernatant was decanted and the cells were re-suspended in 1 mL of Trizol solution (Trizol: DEPC treated water, 1:3). The tubes were mixed thoroughly and left undisturbed for five min at RT for bacterial cell lysis. To the lysed cells, 200 µL of Chloroform was added, mixed and the tubes were left undisturbed for 10 min at RT. The tubes were centrifuged at 12000 g for 15 min at 4°C for the separation of aqueous and organic layer. The aqueous layer was transferred to a fresh tubes containing 500 µL of isopropanol. The tubes were mixed gently and incubated for 30 min for the precipitation of nucleic acids. Precipitated nucleic acids were pelleted by centrifugation at 12000 g for 10 min at 4°C. The pellet was washed with 75% ethanol at 7500 g for 5 min at 4°C air dried and re-dissolved in 20 µL of RNA storage solution (Thermo Fisher Scientific, USA) at 57°C for 10 min, treated with DNase for removal of DNA contamination.

The RNA was purified using DNase I, amplification grade (Thermo Fisher Scientific, USA) as per manufacturers instructions. the reaction mixture for DNase treatment consisted of 1 µg of RNA 1 X DNase I reaction buffer and 1 U of DNase I. the tubes were incubated at RT for 15 min followed by inactivation at 65°C for 10 min by addition of 25

mM EDTA. The RNA was quantified using Nano drop-1000, Thermo fisher scientific, USA. The purified RNA was maintained at -80°C for the synthesis of cDNA.

6.2.9.1. Synthesis of cDNA

The first strand of cDNA was synthesized using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. The reaction mixture for cDNA synthesis was prepared in sterile nuclease free eppendorf tubes on ice. cDNA synthesis was carried out in two steps. In the first step, annealing of the reverse primer to the HA tag sequence was carried out. The reaction mixture was prepared with 1 µg of RNA and 10 mM HAR reverse primer. Annealing was performed at 65°C for 5 min, following which the tubes were maintained on ice. The 20 µL of reaction mixture was completed in the second step by addition of 20 U of Ribolock RNase Inhibitor, 10 mM of dNTPs, and 200 U of Revert Aid M-MuLV Reverse transcriptase (RT). The tubes were mixed gently, centrifuged briefly at 3000 rpm and incubated at 42°C for 60 min. the reaction was terminated at 70°C for 5 min. The incubation steps were performed in Mastercycler Pro (Eppendorf, GmBH).control reaction was performed using 50 $ng.\mu l^{-1}$ of GAPDH RNA and GAPDH reverse primer.

6.2.9.2. Amplification of first strand of cDNA

The transcription of T3E gene RipAM fused to HA tag on C-terminal end was confirmed by PCR of Rs-RipAM and Rs-hrpB-RipAM cDNA. The primers and PCR conditions were same as mentioned for confirmation of the pRCG-Pep-RipAM effector construct from *R. solanacearum* DNA (section 6.2.5.1.1). Five µL of cDNA was used as a template in the reaction mixture instead of template DNA.

6.3. Results and Discussion

The *R. solanacearum* wild type strain Rs-09-161 and its T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$ were used in this study.

6.3.1. Selection of genes for validation as T3E.

Two novel effector genes, *RipAM* and *RS15E* were selected as candidate effectors for validation through secretion studies. These genes were identified as putative T3E genes through bioinformatics analysis (Asolkar and Ramesh, 2018a).

The T3E genes *RipAM* and *RS15E* were amplified with the start codon in frame using a high fidelity, proof reading polymerase. The 3'-end of the genes were amplified without the stop codon. This was necessary for expression of effectors as HA fused proteins at the C terminal end after transfer in the pRCG-Pep-GWY destination vector. The amplified fragments of *RipAM* and *RS15E* are shown in Fig.6.3.

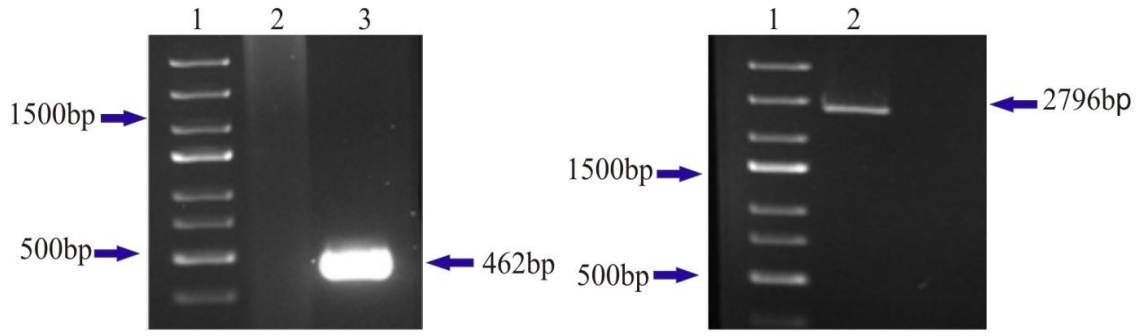


Fig. 6.3.Amplification of T3E genes, *RipAM* and *RS15E*. (A) Amplification of *RipAM* gene. Lane 1: Ladder, lane 2: negative control, lane 3: 462 bp. (B) Amplification of *RS15E* gene. Lane 1: Ladder, lane 2: 2796 bp, lane 3: negative control.

***Ladder:** 100 bp-5 Kb, GeneRuler Express DNA Ladder

6.3.2. Cloning of *RipAM* and *RS15E* in pENTR-SD-D-TOPO

The amplified genes were cloned in the pENTR-SD-D-TOPO vector as per manufacturer's instructions. The pENTR-SD-D-TOPO vector is an entry vector which directionally clones blunt ended PCR products very efficiently for transfer into a GATEWAY vector. The linearized vector is bound to Topoisomerase I from *Vaccinia* virus at the 3'-end of the cloning site through a covalent phospho-tyrosyl bond. The GTGG overhang present on the vector invades 5'-end of the PCR product, anneals to the CACC bases and directs the cloning of the gene is correct orientation.

Colonies grown on LB-Kanamycin (50 µg.mL^{-1}) plates were considered as positive clones. The orientation of effector genes *RipAM* and *RS15E* in the positive colonies were confirmed by colony PCR of Ec-RipAM and Ec-RS15E with gene specific diagnostic primer and M13 reverse primer which gave a characteristic band at 338 bp and 506 bp respectively (Fig. 6.4). The band produced by internal primer and M13 reverse primer also confirmed the correct orientation of the gene with respect to RBS present on the entry vector. The colonies were cultured in LB broth with kanamycin (50 µg.mL^{-1}) and the recombined plasmid was isolated. The isolated plasmid was further confirmed by restriction digestion.

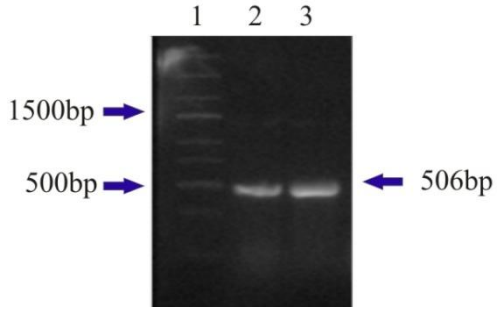


Fig. 6.4.Colony PCR for the confirmation of *RS15E* in the pENTR-SD-D-TOPO vector. Lane 1: ladder; lane 2-3: 506 bp.

Ladder used: 100 bp-5 Kb, Gene Ruler Express DNA ladder

6.3.3. The pRCG-Pep-GWY destination vector

The pRCG-Pep-GWY destination vector was transferred to One Shot® ccdB Survival™ 2 T1R competent cells by heat shock transformation. The pUC19 DNA was used as a control. The transformation efficiency of the pRCG-Pep-GWY was 4.68 x 10^4 and that of pUC 19 was 4.1 x 10^7 CFU.μg^{-1} of plasmid DNA. This vector has the presence of integration element consisting of gentamycin resistance gene and the gateway cassette. The vector is under the control of *Pep* promoter which is active under high cell densities. The gentamycin resistance gene also confers resistance to apramycin sulphate. Since *R. solanacearum* is resistant to gentamycin, apramycin sulphate was used in place of gentamycin.

6.3.4. The LR recombination reaction

The effector genes *RipAM* and *RS15E* were transferred from RipAMpENTR-SD-D-TOPO and RS15EpENTR-SD-D-TOPO to pRCG-Pep-GWY destination vector through LR recombination. This resulted in the formation of pRCG-Pep-RipAM and pRCG-Pep-RS15E. The effector constructs were transferred to *E. coli* S17-1λpir strain leading to the generation of Ec-pRCG-RipAM and Ec-pRCG-RS15E. The confirmation of pRCG-Pep-RipAM and pRCG-Pep-RS15E in Ec-pRCG-RipAM and Ec-pRCG-RS15E is shown in Fig. 6.5. The mechanism by which the lambda phage undergoes excision and integration is used as the principle in the GATEWAY technology. Since it works on the basis of site-directed mutagenesis, it avoids the use of restriction enzymes (Hartley *et al.*, 2000).

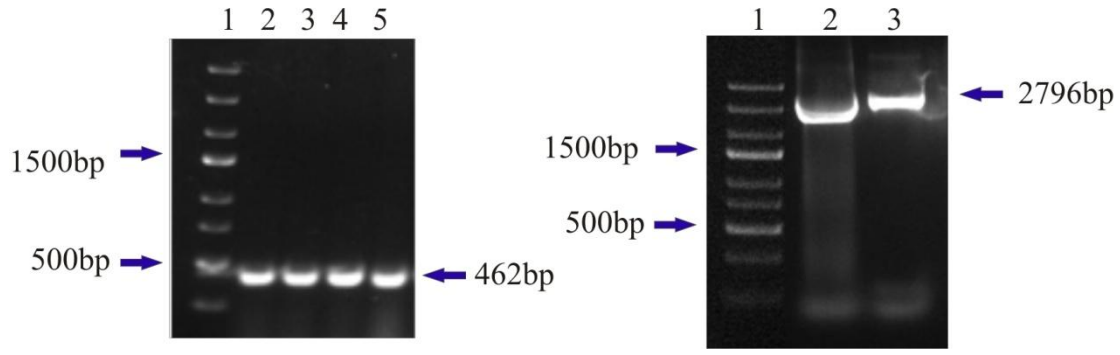


Fig 6.5.Confirmation of *RipAM* and *RS15E* in pRCG-Pep-RipAM and pRCG-Pep-RS15E after LR clonase reaction. (A) Confirmation of *RipAM* in pRCG-Pep-RipAM. Lane 1: ladder; lane 2-5: 462 bp. (B) Confirmation of *RS15E* in pRCG-Pep-RS15E. Lane 1: Control *RS15E* gene; lane 2-3: 2796 bp in *E. coli* colony

Ladder used: 100 bp-5 Kb, Gene Ruler Express DNA Ladder

6.3.5. Transfer of effector constructs in *R. solanacearum*

The effector construct pRCG-Pep-RipAM and pRCG-Pep-RS15E were transferred to *R. solanacearum* strain Rs-09-161 respectively. The pRCG vector series integrates itself stably into the chromosome by double recombination reaction in the pseudogene site of *R. solanacearum* (GMI1000) chromosome (Monteiro *et al.*, 2012a). This eliminates the use of constant selection pressure for maintenance of the vector. PCR performed with gene specific forward primer and the HA reverse primer confirmed the presence of *RipAM* and *RS15E* effector construct fused to HA epitope sequence in Rs-RipAM and Rs-RS15E. The effector constructs pRCG-Pep-RipAM and pRCG-Pep-RS15E were transferred to Rs-hrpB$^-$ and Rs-hrcV$^-$ by natural transformation using the genomic DNA of Rs-RipAM and Rs-RS15E. The confirmation of effector construct in *R. solanacearum* Rs-09-161 and its T3SS mutants, Rs-hrpB- and Rs-hrcV$^-$ is depicted in Fig 6.6.

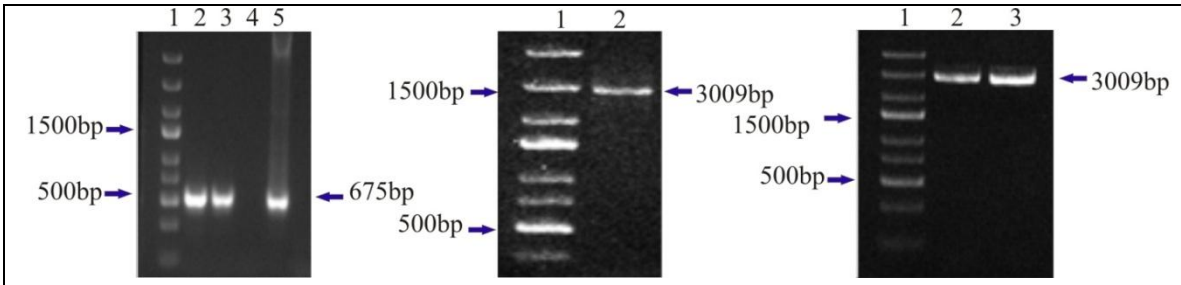


Fig.6.6. Confirmation of pRCG-Pep-RipAM and pRCG-Pep-RS15E in wild type *R. solanacearum* and its T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$. (A) Confirmation of pRCG-Pep-RipAM. Lane1: ladder; lane2: Confirmation in Rs-09-161 (675 bp); lane 3: Confirmation in Rs-hrpB$^-$ (675 bp); lane 5: Confirmation in Rs-hrcV$^-$ (675 bp). (B) Confirmation of pRCG-Pep-RS15E in wild type. Lane1: ladder; lane 2: Confirmation in Rs-09-161 (3009 bp). (C) Confirmation of RS15E in pRCG-Pep-RS15E in T3SS mutants: Rs-hrpB$^-$ and Rs-hrcV$^-$. Lane1: ladder; lane 2: Confirmation in Rs-hrpB$^-$ (3009 bp); lane 3: Confirmation in Rs-hrcV$^-$ (3009 bp).

Ladder used: 100 bp-5 Kb, Gene Ruler Express DNA Ladder

6.3.6. Precipitation of protein

R. solanacearum strain Rs-RipAM, Rs-RS15E and its T3SS mutant counterparts (Rs-hrpB-RipAM and Rs-hrcV-RipAM; Rs-hrpB-RS15E and Rs-hrcV-RS15E respectively) expressing the *RipAM* and *RS15E* gene fused to triple HA tag at the 3'-end were grown to a high cell density in the secretion inducing M63 medium. The total supernatant protein was precipitated by TCA-acetone precipitation and the intracellular protein was obtained by cell lysis (Monteiro *et al.*, 2012a). The supernatant protein was quantified and is in the range of 3-5 mg.mL^{-1}. The TCA-acetone precipitation has been used to precipitate protein with greater efficiency (Hao *et al.*, 2015).

6.3.7. Separation of proteins

The extracellular proteins obtained by TCA-acetone precipitation were separated by SDS-PAGE. The separated proteins were transferred to PVDF membrane for immunodetection of HA fused proteins.

6.3.8. Western Blotting

The immunodetection of HA fused effector protein was performed by Western blotting. Rabbit anti-HA polyclonal primary antibody and alkaline phosphatase conjugated anti-rabbit goat secondary antibody was used in western blotting. Purple color bands were observed on incubation with BCIP/NBT chromogenic substrate. The western blotting was conducted eight times.

The candidate effector Rs_T3E_Hyp15 (referred as 'RS15E') was validated as a T3E by the presence of purple band in the extracellular proteins of Rs-RS15E in between 60 kDA-100 kDA and absence in T3SS mutants, Rs-hrpB-Rs15E and Rs-hrcV-RS15E. However, this band was not detected in the proteins of the bacterial lysates of Rs-RS15E and T3SS mutants, Rs-hrpB-Rs15E and Rs-hrcV-RS15E. The T3E protein RipAM was not detected in the Western blot. This can be probably due to its smaller size or less specificity of the chromogenic detection procedure. Another probable reason can also be that the gene *RipAM* is not under the control of *hrpB* regulation and hence the presence of band in wild type and its absence in T3SS mutants was not visualized. Validation of T3E RS15E is represented in Fig. 6.7.

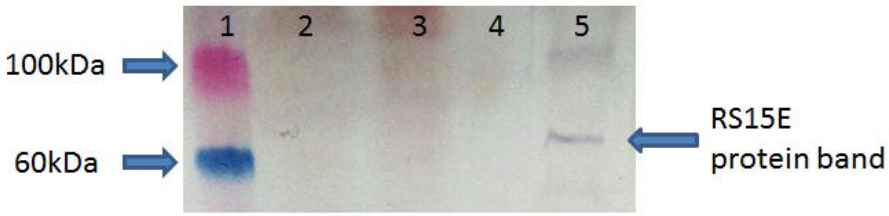


Fig. 6.7.Validation of T3E RS15E in the cell supernantant through Western blotting. Lane 1: ColorBurst Electrophoresis Marker (Mol wt. 8 kDa-22 kDA); lane 2: Rs-hrpB-Rs15E; lane 3: Rs-hrcV-Rs15E; lane 4: not loaded; lane 5-6: Rs-RS15E.

Many T3Es expressed in *R. solanacearum* strains RS1000 and GMI1000 are validated through translocation studies using reporter based systems (Cunnac *et al.,* 2004b; Mukaihara *et al.,* 2009; 2010). Adenylate Cyclase (Cya) reporter system is widely used in *R. solanacearum* for validation of T3E and validates the effector after translocation into the plant cells. Five effector proteins were validated to be translocated into the plants on co-cultivation with *Arabidopsis thaliana* through Cya assays (Cunnac *et al.,* 2004b). Mukaihara and coworkers (2009; 2010) validated 72 T3E in *R. solanacearum* through Cya fused proteins in eggplant. *RipA2* and *RipA4* were validated as T3E by expressing in pRCG-Pep-GWY vector (Monteiro *et al.,* 2012a). The pRCG-Pep-GWY vector is designed especially for detection of T3E in *R. solanacearum*. The vector integrates itself in the genome of *R. solanacearum* through homologous recombination and secretes the T3E gene cloned within it as a protein fused to HA epitope in the C-terminal end (Monteiro *et al.,* 2012a). The HA reporter based method is preferred over the Cya assays as it validates the effectors using *in vitro* methods and eliminates the use of host plants in the validation of T3E. The C-terminal HA tag was used in *Pseudomonas syringae* to study the role of various T3Es (Wei *et al*., 2018). In *Pseudomonas syringae* pv *syringae* B728a

(*Psy*B728a), the T3E HopZ3 was cloned with HA tag to study its role on epiphytic survival and/or growth on *N. Benthamiana* (Lee *et al.*, 2012).

6.3.9. RNA isolation

The T3E gene *RipAM* was not detected in western blotting. Therefore, to check if the RipAM gene is expressed independent of *hrpB*, the mRNA was isolated from the Rs-RipAM and Rs-hrpB-RipAM and the cDNA was synthesized. Amplification of cDNA produced 465bp characteristic band in both the isolates (Fig. 6.8). This proves that the RipAM gene is transcribed in wild type as well as *hrpB* mutant of *R. solanacearum* and hence is not under the control of *hrpB*. Since its transcription is independent of *hrpB*, this is the probable reason for absence of specific band in the wild type. Similar results were also observed by Lonjon and co-workers (2015), wherein the secretion of RipAM was confirmed to be in *hpa* (hypersensitive response and pathogenicity associated) independent manner through proteomic approach.

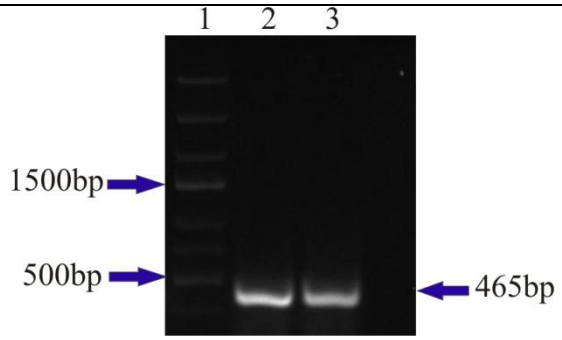


Fig.6.8. Amplification of *RipAM* gene from cDNA in wild type Rs-RipAM and its T3SS mutant Rs-hrpB-RipAM. Lane 1: Ladder; Lane 2; 465 bp in Rs-RipAM; lane 3: 465 bp in Rs-hrpB-RipAM.

Ladder used: 100 bp-5 Kb, Gene Ruler Express DNA Ladder

6.4. Conclusion

Two putative T3E genes *RipAM* and *RS15E* (*Rs_T3E_Hyp15*) were selected for validation through secretion assays. For this purpose the genes were cloned with the ORF and without the stop codon in pRCG-Pep-GWY vector. The pRCG-Pep-GWY vector is specifically designed for validation of T3E genes and provides the cloned gene with C terminal HA tag sequence. The secretion of the HA fused protein was detected by western blotting. RS15E (Rs_T3E_Hyp15) was validated as a T3E as its presence was detected in the wild type and absence in T3SS mutants during western blotting. In case of *RipAM*, the gene was proved to be under *hrp* independent regulation and hence could not be validated through western blot.

CHAPTER VII

Summary

This study was taken up with the aim of analyzing the various virulence factors and T3Es present in *R. solanacearum*. To achieve the results, the study was divided into different parts as follows:

Various virulence genes and T3E genes present in *R. solanacearum* isolates Rs-09-161 and Rs-10-244 were analyzed using bioinformatics methods with the help of reference isolates from different phylotypes. The virulence gene sequences of Rs-09-161 and Rs-10-244 were found to be closely related to GMI1000. The isolates Rs-09-161 and Rs-10-244 were also found to be closely related to Phylotype I, Phylotype III and Phylotype IV isolates.

Seventy two T3Es were identified in Rs-09-161 and 77 in Rs-10-244. Both the isolates, Rs-09-161 and Rs-10-244 had the presence of the T3E gene *RipTPS* in multiple copies. Three candidate effectors (*Rs_T3E_Hyp6, Rs_T3E_Hyp7* and *Rs_T3E_Hyp15*) and three pseudogenes (*RipF1, RipAX1* and *Rs_T3E_Hyp8*) were present in Rs-09-161. One candidate effectors (Rs_T3E_Hyp7) and four pseudogenes (*RipO1, RipAX2, Rs_T3E_Hyp8* and *Rs_T3E_Hyp15*) were present in Rs-09-161. Phylogenetic trees of sequences of effector families RipA, RipG, RipH and RipS resulted in the grouping of the isolates based on their phylotypes. Phylotype I isolates including Rs-09-161 and Rs-10-244 formed a single group. The phylotype III isolate CMR15 formed a group closely associated with phylotype I. The strains belonging to phylotypes II and IV separated to form two different groups.

To find the role of some novel genes in the virulence of *R. solanacearum*, Rs-09-161 was selected as the wild type isolate. Seven genes were selected in Rs-09-161 for development

of mutants. These included two genes *viz.*, *hrpB* and *hrcV* which belong to the type three secretion system (T3SS) and five genes *viz.,tssM, tssH, hcp, vgrG3* and *ompA* genes which belong to the type six secretion system (T6SS). The mutants were developed through insertional mutagenesis by homologous recombination and confirmed with the help of diagnostic primer. Stability studies of the mutants Rs-hrpB$^-$ and Rs-hrcV$^-$ proved them to be stable in the absence of antibiotic for upto a period of 10 days.

The T6SS mutants, Rs-TssM$^-$, Rs-TssH$^-$, Rs-Hcp$^-$, Rs-OmpA$^-$ and Rs-VgrG3$^-$ were used to study the role of T6SS genes in the virulence of *R. solanacearum*with the help of plant assays by petiole inoculation and soil drench inoculation on susceptible tomato, brinjal and moderately resistant tomato. The mutants Rs-VgrG3$^-$ and Rs-OmpA$^-$ were found to be reduced in virulence on its hosts. Rs-VgrG3$^-$ has showed delay to cause 75% wilt on petiole inoculated seedlings of tomato var., *Arka Rakshak* and drench inoculated seedlings of brinjal *cv*., *Agassaim* by two to six days. Rs-OmpA$^-$ delayed the 75% wilt in the petiole inoculated seedlings of *Arka Rakshak* and drench inoculated seedlings of *Agassaim* and *Arka Vikash* by two to four days.

The mutants of the T3SS, Rs-hrpB$^-$ and Rs-hrcV$^-$were developed for the validation of a putative T3E. Apart from this, these mutants were also used for virulence determination on susceptible hosts and were found to be non-pathogenic on susceptible brinjal and tomato in soil drench and petiole inoculation.

The candidate gene *Rs_T3E_Hyp15* was validated as a T3E by detecting its presence in the wild type and absence in the T3SS deficient mutants in Western blotting. The T3E gene *RipAM* was proved to be translocated in *hrp* independent manner.

CHAPTER VIII

Bibliography

Achari, G.A. and Ramesh, R. 2014. Diversity, biocontrol, and plant growth promoting abilities of xylem residing bacteria from solanaceous crops. *International Journal of Microbiology*, Article ID 296521, 14 pages.

Adler, J.1966. Chemotaxis in bacteria. *Science*, **153**:708-716.

Agrios, G.N. 2005. Plant Pathology.5th eds. *Department of Plant Pathology.University of Florida. United States of America*.

Aldon, D., Brito, B., Boucher, C. and Genin, S. 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. *The EMBO Journal*, **19**:2304-2314.

Alfano J.R. and Collmer, A. 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense.*Annual Review Phytopathology*, **42**:385-414.

Allen, C., Gay, J. and Simon-Buela, L. 1997. A regulatory locus, pehSR, controls polygalacturonase production and other virulence functions in *Ralstonia solanacearum*. *Molecular Plant-Microbe Interactions*, **10**:1054-1064.

Álvarez, B., López, M.M. and Biosca, E.G. 2008. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology*, **154**:3590-3598.

Alvarez, B., Biosca, E. and Lopez, M. 2010. On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* Ed. A. Mendez Vilaspp. vol. 1 pp 267-279. Formatex, Badajoz, Spain.

Animal and Plant Health Inspection Service U. (2002) Agricultural Bioterrorism Protection Act of 2002; Possession, use, and transfer of biological agents and toxins, National Archives and Records administration, Vol. 67, pp. 76908-76938.

Anith, K.N., Momol, M.T., Kloepper, J.W., Marois, J.J., Olson, S.M. and Jones, J.B. 2004. Efficacy of plant growth promoting rhizobacteria, acibenzolar-S-methyl and soil amendment for integrated management of bacterial wilt on tomato.*Plant Disease*, **88**:669-673.

Araud-Razou, I., Vasse, J., Montrozier, H., Etchebar, C. and Trigalet, A. 1998. Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum*

and its role in tomato root infection and vascular colonization. *European Journal of plant pathology*, **104**:795-809.

Aschtgen, M.S., Bernard, C.S., De Bentzmann, S., Lloubes, R. and Cascales, E. 2008. SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *Journal of Bacteriology*, **190 :**7523-7531.

Aschtgen, M. S., Gavioli, M., Dessen, A., Lloubes, R. and Cascales, E. 2010. The SciZ protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the cell wall. *Molecular Microbiology*, **75**:886–899.

Asolkar, T. and Ramesh, R. 2018a. Identification of virulence factors and type III effectors of phylotype I, Indian *Ralstonia solanacearum* strains Rs-09-161 and Rs-10-244. *Journal of Genetics*, **97**:55-66.

Asolkar, T. and Ramesh, R.2018b. Development of T3SS Mutants (hrpB$^-$ and hrcV$^-$) of *Ralstonia solanacearum*, Evaluation of Virulence Attenuation in Brinjal and Tomato-A Pre-requisite to Validate T3Es of *R. solanacearum*. *Indian Journal of Microbiology*, **58**:372-380.

Assefa, M., Dawit, W., Lencho, A. and Hunduma, T. 2015. Assessment of wilt intensity and identification of causal fungal and bacterial pathogens on hot pepper (*Capsicum annuum* L.) in BakoTibbe and Nonno districts of west Shewa zone, Ethiopia. *International Journal of Phytopathology*, **4**:21-28.

Badr, S., Li, Y. and Duan, K. 2016. Comparison of the Structure, Regulation and Functions between Type Three and Type Six Secretion System in Gram-Negative Bacteria. *Journal of Medical Microbiology and Diagnosis*, **5**:2161-0703.

Bahassi, E. M., O'Dea, M. H., Allali, N., Messens, J., Gellert, M. and Couturier, M. 1999. Interactions of CcdB with DNA gyrase.Inactivation of Gyra, poisoning of the gyrase-DNA complex, and the antidote action of CcdA.*The Journal of Biological Chemistry*, **274**:10936–10944.

Basler, Á., Pilhofer, Á., Henderson, G.P., Jensen, G.J. and Mekalanos, J.J. 2012. Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature*, **483**:182.

Bholanath, M., Indrabrata, B. and Khatua, D.C. 2014. Incidence of bacterial wilt disease in West Bengal, India. *Academia Journal of Agricultural Research*, **2**:139-146.

Blair, D.F. 1995. How bacteria sense and swim. *Annual Reviews in Microbiology*, **49**:489-520.

Boucher, C. A., Van Gijsegem, F., Barberies, P. A., Arlat, M. and Zischek, C. 1987. *Pseudomanas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *Journal of Bacteriology*, **169**:5626-5633.

Boucher, C., Barberis, P., Trigalet, A. and Demery D. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn 5 -induced avirulent mutants. *Journal of General Microbiology*, **131**:2449-2457.

Boucher, C.A., Barberis, P.A. and Arlat, M. 1988. Acridine Orange Selects for Deletion of *hrp* Genes. *Molecular Plant Microbe Interactions*, **1**:282-288.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y. and Attree, I. 2009. Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources?. *BMC genomics*, **10**:104.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**:248-254.

Bretz, J.R. and Hutcheson, S.W. 2004. Role of type III effector secretion during bacterial pathogenesis in another kingdom. *Infection and immunity*, **72**:3697-3705.

Brito, B., Aldon, D., Barberis, P., Boucher, C. and Genin, S. 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum* hrp genes. *Molecular Plant-Microbe interactions*, **15**:109-119.

Brito, B., Marenda, M., Barberis, P., Boucher, C. and Genin, S. 1999. *prhJ* and *hrpG*, two new members of the plant signal-dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. *Molecular Microbiology*, **31**:237-251.

Brooks, T.M., Unterweger, D., Bachmann, V., Kostiuk, B. and Pukatzki, S. 2013. Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *Journal of Biological Chemistry*, **288**:7618-7625.

Brumbley, S.M. and Denny, T.P. 1990. Cloning of wild-type *Pseudomonas solanacearum* phcA, a gene that when mutated alters expression of multiple traits that contribute to virulence. *Journal of Bacteriology*, **172**:5677-5685.

Brumbley, S.M., Carney, B.F. and Denny, T.P. 1993.Phenotype conversion in Pseudomonas solanacearum due to spontaneous inactivation of PhcA, a putative LysR transcriptional regulator. *Journal of Bacteriology*, **175**:5477-5487.

Brunet, Y.R., Zoued, A., Boyer, F., Douzi, B. and Cascales, E. 2015. The type VI secretion TssEFGK-VgrG phage-like baseplate is recruited to the TssJLM membrane complex via multiple contacts and serves as assembly platform for tail tube/sheath polymerization. *PLoS Genetics*, **11**:e1005545.

Buddenhagen, I.W. and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*.*Annual Review of Phytopathology*, **2**:203-230.

Buddenhagen, I.W. 1986. Bacterial wilt disease in Asia and the South Pacific. *In*: Persley, G.J. (ed.) *Proceedings of an International Workshop ACIAR Proceedings* pp126-143, Philippines.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A. and Hooykaas, P.J. 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *The EMBO Journal*, **14**:3206-14.

Cascales, E. and Cambillau, C. 2012. Structural biology of type VI secretion systems. *Philosophical Transactions of the Royal Society B*, **367**:1102-1111.

Cascales, E. and Christie, P.J. 2003.The versatile bacterial type IV secretion systems.*Nature Reviews. Microbiology*, **1**:137-49.

Champoiseau, P.G., Jones, J.B. and Allen, C. 2009. *Ralstonia solanacearum* race 3 biovar 2 causes tropical losses and temperate anxieties. *Plant Health Progress*, **10***:*1-10.

Chandrashekara, K. N. and Prasannakumar M.K. 2010. New host plants for *Ralstonia solanacearum* from India. *Plant Pathology*, **59**:1164-1164.

Chandrashekara, K., Prasannakumar, M., Deepa, M., Vani, A. and Khan, A. 2012.Prevalence of races and biotypes of *Ralstonia solanacearum* in India. *Journal of Plant Protection Research*, **52:**53-58.

Chen, L., Shirota, M., Zhang, Y., Kiba, A., Hikichi, Y. and Ohnishi, K. 2014.Involvement of HLK effectors in *Ralstonia solanacearum* disease development in tomato. *Journal of General Plant Pathology*, **80**:79-84.

Chen, Y., He, L., and Xu, J. 2005.Detection of bacterial wilt infection in potato using PCR.*Journal of Plant Protection Research*, **32**:129–132.

Choi, C.H., Lee, J.S., Lee, Y.C., Park, T.I. and Lee, J.C. 2008.*Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC Microbiology*, **8**:216

Cianciotto, N.P. 2013. Type II secretion and Legionella virulence. *Current Topics in Microbiology and Immunology*, **376**:81-102.

Clough, S. J., Flavier, A. B., Schell, M. A. and Denny, T. P. 1997a. Differential expression of virulence genes and motility in *Ralstonia (Pseudomonas) solanacearum* during exponential growth. *Applied and Environmental Microbiology*, **63**:844-850.

Clough, S. J., Lee, K. E., Schell, M. A. and Denny, T. P. 1997b. A two component system in Ralstonia (Pseudomonas) solanacearum modulates production of PhcA-regulated virulence factors in response to 3-hydroxypalmitic acid methyl ester. *Journal of Bacteriology*, **179**:3639-3648.

Coburn, B., Sekirov, I. and Finlay, B. B. 2007. Type III Secretion Systems and Disease. *Clinical Microbiology Reviews*, **20**:535-549.

Coll, N.S. and Valls, M. 2013. Current knowledge on the *Ralstonia solanacearum* type III secretion system. *Microbial Biotechnology*, **6**:614-620.

Cornelis, G.R. 2006. The type III secretion injectisome. *Nature Reviews Microbiology*, **4:**811.

Cui, F., Wu, S., Sun, W., Coaker, G., Kunkel, B., He, P. and Shan, L. 2013. The *Pseudomonas syringae* type III effector AvrRpt2 promotes pathogen virulence via stimulating Arabidopsis auxin/indole acetic acid protein turnover. *Plant Physiology*, **162**:1018-1029.

Cunnac, S., Boucher, C. and Genin, S. 2004a. Characterization of the cis-acting regulatory element controlling HrpB-mediated activation of the type III secretion system and effector genes in *Ralstonia solanacearum*. *Journal of Bacteriology*, **186**:2309-2318.

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C. and Genin, S. 2004b. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*:

identification of novel effector proteins translocated to plant host cells through the type III secretion system. *Molecular Microbiology*, **53**:115-28.

Dalal, N.R., Dalal, S.R., Golliwar, V.G. and Khobragade, R.I. 1999. Studies on grading and pre-packaging of some bacterial wilt resistant brinjal (*Solanum melongena* L.) varieties. *Journal of Soils and Crops*, **9**:223-226.

Delepelaire, P., 2004. Type I secretion in gram-negative bacteria. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, **1694**:149-161.

Denny, T. and Hayward, A.C. 2001. Laboratory guide for identification of plant pathogenic bacteria. *174p, American Phytopathological Society Press. St. Paul, Minn., USA.*

Denny, T. P. 2006. Plant pathogenic *Ralstonia* species, *In Plant-associated bacteria* ed. S. S. Gnanamanickampp 573-644. Springer Netherlands.

Dolores, J.S., Agarwal, S., Egerer, M. and Satchell, K.J. 2015. *Vibrio cholerae* MARTX toxin heterologous translocation of beta-lactamase and roles of individual effector domains on cytoskeleton dynamics.*Molecular Microbiology*, **95** :590-604.

Elphinstone, J.G. 1996. Survival and possibilities for extinction of *Pseudomonas solanacearum* (Smith) Smith in cool climates. *Potato Research*, **39:**403-410.

Elphinstone, J.G. 2005. The current bacterial wilt situation: a global overview. *Bacterial wilt disease and the Ralstonia solanacearum species complex*, 9-28.

Elsas, J.D.V., Kastelein, P., de Vries, P.M. and van Overbeek, L.S. 2001. Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum*bv. 2 in irrigation water. *Canadian Journal of Microbiology*, **47**:842-854.

Engelbrecht-Wiggans, R. 1994. Sequential auctions of stochastically equivalent objects. *Economics Letters*, **44**:87-90.

EPPO/CABI 1996. *Ralstonia solanacearum*. In: Quarantine pests for Europe. 2nd edition Ed. Smith, I.M., McNamara, D.G., Scott, P.R. and Holderness, M. CAB International, Wallingford, UK

EPPO, 2004. Diagnostic protocols for regulated pests: *Ralstonia solanacearum*. Bulletin OEPP/EPPO Bulletin **34**: 173- 178.

Etchebar, C., Trigalet-Demery, D., van Gijsegem, F., Vasse, J. and Trigalet, A. 1998. Xylem colonization by an HrcV mutant of *Ralstonia solanacearum* is a key factor for the efficient biological control of tomato bacterial wilt. *Molecular Plant-Microbe Interactions*, **11**:869-877.

Fegan, M. and Prior, P. 2005. *How complex is the Ralstonia solanacearum species complex*. In *Bacterial wilt disease and the Ralstonia solanacearum species complex*, eds. Allen C., Prior P., and Hayward A. C. pp. 449-461. APS press, Madison, Wisconsin, USA

Felisberto-Rodrigues, C., Durand, E., Aschtgen, M.S., Blangy, S., Ortiz-Lombardia, M., Douzi, B., Cambillau, C. and Cascales, E. 2011. Towards a structural comprehension of bacterial type VI secretion systems: characterization of the TssJ-TssM complex of an Escherichia coli pathovar. *PLoS Pathogens*, **7**:e1002386.

Fenselau, S. and Bonas, U. 1995. Sequence and expression analysis of the hrpB pathogenicity operon of *Xanthomonas campestris pv. vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. *Molecular Plant Microbe Interactions*, **8**:845-854.

Flavier, A. B., Clough, S. J., Schell, M. A. and Denny, T. P. 1997. Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*.*Molecular Microbiology*, **26**:251-259.

Foissac, S., Gouzy, J., Rombauts, S., Mathé, C., Amselem, J., Sterck, L., de Peer, Y.V., Rouzé, P. and Schiex, T. 2008. Genome annotation in plants and fungi: EuGene as a model platform. *Current Bioinformatics*, **3**:87-97.

Gallique, M., Decoin, V., Barbey, C., Rosay, T., Feuilloley, M.G., Orange, N. and Merieau, A. 2017. Contribution of the *Pseudomonas fluorescens* MFE01 type VI secretion system to biofilm formation. *PloS One*, **12**:e0170770.

Genin S., Gough C. L., Zischek C. and Boucher C. A. 1992.Evidence that the hrpB gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Molecular Microbiology*, **6**:3065-3076.

Genin, S. and Boucher, C. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. *Annual Review of Phytopathology*, **42**:107-134.

Genin, S. and Denny, T. P. 2012. Pathogenomics of the *Ralstonia solanacearum* species complex.*Annual Review Phytopathology*, **50**:67-89.

Genin, S., Brito, B., Denny, T.P. and Boucher, C. 2005. Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Letters*, **579**:2077-2081.

González, E.T. and Allen, C. 2003. Characterization of a *Ralstonia solanacearum* operon required for polygalacturonate degradation and uptake of galacturonic acid. *Molecular Plant Microbe Interactions*, **16**:536-544.

Graham, J., Jones, D.A. and Lloyd, A.B. 1979. Survival of *Pseudomonas solanacearum* Race 3 in plant debris and in latently infected potato tubers. *Phytopathology*, **69**:1100-1103

Green, E.R. and Mecsas, J. 2016. Bacterial secretion systems–an overview. *Microbiology Spectrum*, **4**:1-32

Grey, B.E. and Steck, T.R. 2001. The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Applied and Environmental Microbiology*, **67**:3866-3872.

Hajri, A., Pothier, J. F., Fischer-Le Saux, M., Bonneau, S., Poussier, S. *et al*. 2011. Type three effector genes distribution and sequence analysis provides new insights into pathogenicity of plant pathogenic *Xanthomonas arboricola*. *Applied and Environmental Microbiology*, **78**:371-384.

Hao, R., Adoligbe, C., Jiang, B., Zhao, X., Gui, L., Qu, K., Wu, S. and Zan, L. 2015. An Optimized trichloroacetic acid/acetone precipitation method for two-dimensional gel electrophoresis analysis of Qinchuan cattle Longissimus dorsi muscle containing high proportion of marbling. *PloS one*, **10**:e0124723.

Hann, D.R., Dominguez-Ferreras, A., Motyka, V., Dobrev, P.I., Schornack, S., Jehle, A., Felix, G., Chinchilla, D., Rathjen, J.P. and Boller, T. 2013. The *Pseudomonas* type III effector HopQ1 activates cytokinin signaling and interferes with plant innate immunity. *New Phytologist*, **201**: 585-598.

Hartley, J.L., Temple, G.F. and Brasch, M.A. 2000. DNA cloning using in vitro site-specific recombination. *Genome Research*, **10**:1788-1795.

Hayward, A. 1964. Characteristics of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology*, **27**:265-277.

Hayward, A.C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*.*Annual Review of Phytopathology*, **29**:65-87.

Hayward, AC. 1994. The hosts of *Pseudomonas solanacearum*. In Bacterial Wilt: The Disease and Its Causative Agent, *Pseudomonas solanacearum*. Eds. Hayward, A.C and Hartman, G.L. CAB International pp9-24 Wallingford, .

Ho, Y.P., Tan, C.M., Li, M.Y., Lin, H., Deng, W.L. and Yang, J.Y. 2013. The AvrB_AvrC domain of AvrXccC of *Xanthomonas campestris pv. campestris* is required to elicit plant defense responses and manipulate ABA homeostasis. *Molecular Plant Microbe Interactions*, **26**:419-430.

Hong, J.C., Momol, M.T., Jones, J.B., Ji, P., Olson, S.M., Allen, C., Perez, A., Pradhanang, P. and Guven, K. 2008. Detection of *Ralstonia solanacearum* in irrigation ponds and aquatic weeds associated with the ponds in North Florida. *Plant Disease*, **92**:1674-1682.

Hua, Z. and Vierstra, R.D. 2011.The cullin-RING ubiquitin-protein ligases. *Annual Review of Plant Biology*, **62**:299-334.

Huang, Q. and Allen, C. 2000. Polygalacturonases are required for rapid colonization and full virulence of *Ralstonia solanacearum* on tomato plants. *Physiological and Molecular Plant Pathology*, **57**:77-83.

Hueck, C.J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews*, **62**:379-433.

Hughes, C., Muller, D., Hackerm, J. and Goebel, W. 1982.Genetics and pathogenic role of *Escherichia coli* haemolysin. *Toxicon*, **20**:247-52.

Islam, T.M. and Toyota, K. 2004. Suppression of bacterial wilt of tomato by *Ralstonia solanacearum* by incorporation of composts in soil and possible mechanisms. *Microbes and Environments*, **19**:53-60.

Jacobs, J.M., Babujee, L., Meng, F., Milling, A., Allen, C. (2012) The*in planta* transcriptome of *Ralstonia solanacearum*: conserved physiological and virulence strategies during bacterial wilt of tomato. *MBio*, **3**:00114-12.

Jia, S., Qu, X., Feng, L., Tang, T., Tang, Y., Liu, K., Zheng, P., Zhao, Y., Bai, Y. and Cai, M. 1999. Expression of antibacterial peptide gene in transgenic potato confers resistance to bacterial wilt. *Chinese Agricultural Sciences, Beijing, China*, 15-22.

Jiang, B. and Howard, S.P. 1992. The *Aeromonas-Hydrophila* Exee Gene, Required Both for Protein Secretion and Normal Outer-Membrane Biogenesis, Is a Member of a General Secretion Pathway. *Molecular Microbiology*, **6**:1351-1361.

Jukes, T. H. and Cantor, C. R. 1969. Evolution of protein molecules. In *Mammalian Protein Metabolism, ed. H. N.* Munro, pp. 21-132. Academic Press, New York.

Kanamaru, S. 2009. Structural similarity of tailed phages and pathogenic bacterial secretion systems. *Proceedings of the National Academy of Sciences*, **106**:4067-4068.

Kang, Y., Liu, H., Genin, S., Schell, M.A. and Denny, T.P. 2002. *Ralstonia solanacearum*requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. *Molecular Microbiology,* **46**:427-437.

Kapitein, N., Bönemann, G., Pietrosiuk, A., Seyffer, F., Hausser, I., Locker, J.K. and Mogk, A. 2013. ClpV recycles VipA/VipB tubules and prevents non-productive tubule formation to ensure efficient type VI protein secretion. *Molecular Microbiology*, **87**:1013-1028.

Kassa, B., Kifelew, H., Hunduma, T. and Sadessa K. 2015.Status of Ginger Wilt and Identification of the Causal Organism in Southern Nations Nationality and People Sates of Ethiopia.*International Journal of Research Studies in Agricultural Sciences,* **4** :1-11.

Kelman, A., 1953. The bacterial wilt caused by *Pseudomonas solanacearum*. A literature review and bibliography. *North Carolina Agricultural Experimental Station Technical Bulletin,* **99**:194.

Kelman, A. 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in a tetrazolium medium. *Phytopathology*, **44**:693-695.

Korotkov, K.V., Sandkvistm, M. and Hol, W.G.J. 2012. The type II secretion system: biogenesis, molecular architecture and mechanism. *Nature Reviews Microbiology*, **10**:336-351.

Kumar, P. and Sood, A.K. 2001. Integration of antagonistic rhizobacteria and soil solarization for the management of bacterial wilt of tomato caused by *Ralstonia solanacearum.Indian Phytopathology*, **5**4:12-15.

Kumar, A., Sarma, Y.R. and Anandaraj, M. 2004. Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using REP–PCR and PCR–RFLP. *Current Science*, 1555-1561.

Kumar, A., Prameela, T.P., Bhai, R.S., Siljo, A., Biju, C.N., Anandaraj, M. and Vinatzer, B.A. 2012. Small cardamom (*Elettaria cardamomum Maton*.) and ginger (*Zingiber officinale Roxb*) bacterial wilt is caused by same strain of *Ralstonia solanacearum*: a result revealed by multilocus sequence typing (MLST). *European Journal of Plant Pathology*, **132**:477-482.

Kumar, A., Prameela, T.P., Suseelabhai, R., Siljo, A., Anandaraj, M. and Vinatzer, B.A. 2014. Host specificity and genetic diversity of race 4 strains of *Ralstonia solanacearu*m. *Plant Pathology*, **63**:1138-1148.

Kumar, A., Munjal, V., Sheoran, N., Prameela, T.P., Suseelabhai, R., Aggarwal, R., Jain, R.K. and Eapen, S.J. 2017. Draft Genome Sequence of Highly Virulent Race 4/Biovar 3 of *Ralstonia solanacearum* CaRs_Mep Causing Bacterial Wilt in Zingiberaceae Plants in India. *Genome Announcements*, **5**:01420-16.

Lee, J.S., Choi, C.H., Kim, J.W. and Lee, J.C. 2010. *Acinetobacter baumannii* outer membrane protein A induces dendritic cell death through mitochondrial targeting. *The Journal of Microbiology*, **48**:387-392.

Lee, J., Teitzel, G.M., Munkvold, K., del Pozo, O., Martin, G.B., Michelmore, R.W. and Greenberg, J.T. 2012. Type III secretion and effectors shape the survival and growth pattern of *Pseudomonas syringae* on leaf surfaces. *Plant physiology*, **161**:2049-2061.

Letoffe, S., Delepelaire, P. and Wandersman, C. 1996. Protein secretion in gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding. *The EMBO Journal*, **15**:5804-11.

Liu, H., Kang, Y., Genin, S., Schell, M.A. and Denny, T.P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. *Microbiology*, **147**:3215-3229.

Liu, H., Zhang, S., Schell, M.A. and Denny, T.P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. *Molecular Plant–Microbe Interactions,* **18**:1296-1305.

Logger, L., Aschtgen, M.-S., Guérin, M., Cascales, E., and Durand, E. 2016. Molecular dissection of the interface between the type VI secretion TssM cytoplasmic domain and the TssG baseplate component.*Journal of Molecular Biology*, **428**:4424–4437.

Lonjon, F., Turner, M., Henry, C., Rengel, D., Lohou, D., van de Kerkhove, Q., Cazalé, A.C., Peeters, N., Genin, S. and Vailleau, F. 2015. Comparative secretome analysis of *Ralstonia solanacearum* Type 3 secretion-associated mutants reveals a fine control of effector delivery, essential for bacterial pathogenicity. *Molecular and Cellular Proteomics*, **15**:598-613.

Lossi, N.S., Manoli, E., Forster, A., Dajani, R., Pape, T., Freemont, P. and Filloux, A. 2013. The HsiB1C1 (TssB/TssC) complex of the *Pseudomonas aeruginosa* type VI secretion system forms a bacteriophage tail sheath-like structure. *Journal of Biological Chemistry*, **288**:7536–7548.

Lu, H.M. and Lory, S. 1996. A specific targeting domain in mature exotoxin A is required for its extracellular secretion from *Pseudomonas aeruginosa*. *The EMBO Journal,* **15**:429-436.

Ma, L. S., Lin, J. S. and Lai, E. M. 2009. An IcmF family protein, ImpLM, is an integral inner membrane protein interacting with ImpKL, and its Walker A motif is required for type VI secretion system-mediated Hcp secretion in *Agrobacterium tumefaciens. Journal of Bacteriology,* **191**:4316-4329.

Macho, A.P. 2016. Subversion of plant cellular functions by bacterial type-III effectors: beyond suppression of immunity. *New Phytologist*, **210**:51-57.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M.A.X., Verdier, V., Beer, S.V., Machado, M.A. and Toth, I.A.N. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, **13**:614-629.

Marenda, M., Brito, B., Callard, D., Genin, S., Barberis, P., Boucher, C and Arlat, M. 1998. PrhA controls a novel regulatory pathway required for the specific induction

of *Ralstonia solanacearum hrp* genes in the presence of plant cells. *Molecular Microbiology*, **27**:437-453.

Marois, E., Van den Ackerveken, G. and Bonas, U. 2002. The *xanthomonas* type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. *Molecular Plant–Microbe Interactions,* **15**:637-646.

Matsuda, Y., Toyoda, H., Yasunari, K.A.T.O., Kakutani, K., Nakanishi, T., Bingo, M., Nonomura, T. and Ouchi, S., 2000. Molecular monitoring of hrpB-disrupted Mutant of *Ralstonia solanacearum* in tomato plants. *Journal of General Plant Pathology*, **66**:59-63.

Meng, F., Yao, J. and Allen, C. 2011. A MotN mutant of *Ralstonia solanacearum* is hypermotile and has reduced virulence. *Journal of Bacteriology*, **193**:2477-2486.

Minamoto, T., Wada, E. and Shimizu, I. 2012.A new method for random mutagenesis by error-prone polymerase chain reaction using heavy water. *Journal of Biotechnology*, **157 :**71-74.

Miyata, S.T., Bachmann, V. and Pukatzki, S. 2013.Type VI secretion system regulation as a consequence of evolutionary pressure. *Journal of Medical Microbiology*, **62**:663-676.

Mole, B.M., Baltrus, D.A., Dangl, J.L. and Grant, S.R. 2007. Global virulence regulation networks in phytopathogenic bacteria. *Trends in Microbiology*, **15**:363-371.

Monteiro, F., Sole, M., Van Dijk, I. and Valls, M. 2012a. A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in *Ralstonia solanacearum. Molecular Plant Microbe Interactions,* **25**:557-568.

Monteiro, F., Genin, S., Van Dijk, I. and Valls, M. 2012b. A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection. *Microbiology,* **158**:2107-2116.

Mukaihara, T. and Tamura, N. 2009. Identification of novel *Ralstonia solanacearum*type III effector proteins through translocation analysis of *hrpB*-regulated gene products. *Microbiology*, **155**:2235-2244.

Mukaihara, T., Tamura, N. and Iwabuchi, M. 2010. Genome-Wide Identification of a Large Repertoire of *Ralstonia solanacearum* type IIIeEffector proteins by a new functional screen. *Molecular Plant Microbe Interactions*, **23**:251–262.

Mukaihara, T., Tamura, N., Murata, Y. and Iwabuchi, M. 2004.Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*. *Molecular Microbiology*, **54**:863-875.

Nion, Y.A. and Toyota, K. 2015. Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*. *Microbes and Environments*, **30**:1-11.

Noel, L., Thieme, F., Gäbler, J., Büttner, D. and Bonas, U. 2003. XopC and XopJ, two novel type III effector proteins from *Xanthomonas campestris pv. vesicatoria*. *Journal of Bacteriology*, **185**:7092-7102.

Orgambide, G., Montrozier, H., Servin, P., Roussel, J., Trigalet-Demery, D. and Trigalet, A. 1991. High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum* strain GMI 1000 and the complete structure of the major polysaccharide. *Journal of Biological Chemistry*, **266**:8312-8321.

Patil, V.U., Girimalla, V., Sagar, V., Chauhan, R.S. and Chakrabarti, S.K. 2017. Genome sequencing of four strains of Phylotype I, II and IV of *Ralstonia solanacearum* that cause potato bacterial wilt in India. *Brazilian Journal of Microbiology*, **48**:193-195.

Pautsch, A. and Schulz, G.E. 1998. Structure of the outer membrane protein A transmembrane domain. *Nature Structural and Molecular Biology*, **5**:1013–1017.

Peeters, N., Carrere, S., Anisimova, M., Plener, L., Cazale, A.C. and Genin, S. 2013. Repertoire, unified nomenclature and evolution of the type III effector gene set in the *Ralstonia solanacearum* species complex. *BMC Genomics*, **14**:859-877.

Peng, Y., Wang, X., Shou, J., Zong, B., Zhang, Y., Tan, J., Chen, J., Hu, L., Zhu, Y., Chen, H. and Tan, C. 2016. Roles of Hcp family proteins in the pathogenesis of the porcine extraintestinal pathogenic *Escherichia coli* type VI secretion system. *Scientific Reports*, *6*, p.26816.

Persson, P. 1998. Successful eradication of *Ralstonia solanacearum* from Sweden 1. *EPPO Bulletin*, **28**:113-119.

Plener, L., Manfredi, P., Valls, M. and Genin. S. 2010. PrhG, a transcriptional regulator responding to growth conditions, is involved in the control of the type III secretion system regulon in *Ralstonia solanacearum*. *Journal of Bacteriology*, **192**:1011-9.

Poueymiro, M. and Genin, S. 2009. Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. *Current Opinion in Microbiology*, **12**:44–52

Poueymiro, M., Cazale, A.C., Francois, J.M., Parrou, J.L., Peeters, N. and Genin, S. 2014. A *Ralstonia solanacearum* type III effector directs the production of the plant signal metabolite trehalose-6-phosphate. *MBio*, **5**:02065-14.

Poussier, S., Thoquet, P., Trigalet-Demery, D., Barthet, S., Meyer, D., Arlat, M. and Trigalet, A. 2003.Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from non-pathogenic to pathogenic forms via alterations in the phcA gene. *Molecular Microbiology*, **49**:991-1003.

Pradhanang, P.M., Momol, M.T., Olson, S.M. and Jones, J.B. 2003. Effects of plant essential oils on *Ralstonia solanacearum* population density and bacterial wilt incidence in tomato. *Plant Disease*, **87**:423-427.

Prior, P., Ailloud, F., Dalsing, B.L., Remenant, B., Sanchez, B. and Allen, C. 2016. Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. *BMC Genomics*, **17**:90-111.

Puhar, A. and Sansonetti, P.J. 2014. Type III secretion system. *Current Biology*, **24**:784-791.

Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., Heidelberg, J.F. and Mekalanos, J.J. 2006. Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences*, **103**:1528-1533.

Pukatzki, S., McAuley, S.B. and Miyata, S.T. 2009. The type VI secretion system: translocation of effectors and effector-domains. *Current Opinion in Microbiology*, **12**:11-17.

Ramesh, R. and Phadke, G.S. 2012. Rhizosphere and endophytic bacteria for the suppression of eggplant wilt caused by *Ralstonia solanacearum*. *Crop Protection*, **37***:*35-41.

Ramesh, R., Achari, G., Asolkar, T., Dsouza, M. and Singh, N. 2016. Management of bacterial wilt of brinjal using wild brinjal (*Solanum torvum Sw*) as root stock. *Indian Phytopathology*, **69**:2-6.

Ramesh, R., Achari, G.A. and Gaitonde, S. 2014a. Genetic diversity of *Ralstonia solanacearum* infecting solanaceous vegetables from India reveals the existence of unknown or newer sequevars of Phylotype I strains. *European Journal of Plant Pathology*, **140**:543-562.

Ramesh, R., Gaitonde, S., Achari, G., Asolkar, T., Singh, N.P., Carrere, S. *et al*. 2014b. Genome Sequencing of *Ralstonia solanacearum*Biovar 3, Phylotype I, Strains Rs-09-161 and Rs-10-244, Isolated from Eggplant and Chili in India. *Genome Announcement*, **2**:1-2.

Records, A. R. 2011. The Type VI secretion system: a multipurpose delivery system with a phage like machinery. *Molecular Plant Microbe Interactions*, **24**:751-757.

Remenant, B., de Cambiaire, J.C., Cellier, G., Jacobs, J.M., Mangenot, S., Barbe, V., Lajus, A., Vallenet, D., Medigue, C., Fegan, M. and Allen, C. 2011. *Ralstonia syzygii*, the blood disease bacterium and some Asian *R. solanacearum* strains form a single genomic species despite divergent lifestyles. *PLoS One*, **6**:e24356.

Remigi, P., Anisimova, M., Guidot, A., Genin, S. and Peeters, N. 2011. Functional diversification of the GALA type III effector family contributes to *Ralstonia solanacearum* adaptation on different plant hosts. *New Phytologist*, **192**:976-987.

Safni, I., Cleenwerck, I., De Vos, P., Fegan, M., Sly, L. and Kappler, U. 2014.Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **64**:3087-3103.

Sagar, V., Jeevalatha, A., Mian, S., Chakrabarti, S.K., Gurjar, M.S., Arora, R.K., Sharma, S., Bakade, R.R. and Singh, B.P. 2014. Potato bacterial wilt in India caused by

strains of phylotype I, II and IV of *Ralstonia solanacearum*. *European Journal of Plant Pathology*, **138**:51-65.

Saile, E., McGarvey, J., Schell, M. and Denny, T. 1997.Role of Extracellular Polysaccharide and Endoglucanase in Root Invasion and Colonization of Tomato Plants by *Ralstonia solanacearum*. *Phytopathology*, **87**:1264-1271.

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M. *et al*. 2002 Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature*, **415**:497-502.

Sambrook, J. and Russell D.W. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York, USA.

Sandkvist, M., Michel, L.O., Hough, L.P., Morales, V.M., Bagdasarian, M., Koomey, M., DiRita, V.J. and Bagdasarian, M. 1997. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. *Journal of Bacteriology*, **179** :6994-7003.

Schaad, N. W., Jones, J.B. and Chun, W. Laboratory Guide for Identification of Plant Pathogenic Bacteria **(**third ed.), American Phytopathological Society Press, pp. 155-157, Minnesota, USA

Schell M.A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum*by an elaborate sensory network. *Annual Review Phytopathology*, **38**:263-292.

Shrivastava, S. and Mande, S.S. 2008. Identification and functional characterization of gene components of Type VI Secretion system in bacterial genomes. *PloS one*, **3**:e2955.

Silverman, J.M., Brunet, Y.R., Cascales, E. and Mougous, J.D. 2012. Structure and regulation of the type VI secretion system. *Annual Review of Microbiology*, **66**:453-472.

Singh, D., Yadav, D.K., Sinha, S. and Upadhyay, B.K. 2012. Utilization of plant growth promoting Bacillus subtilis isolates for the management of bacterial wilt incidence in tomato caused by *Ralstonia solanacearum* race 1 biovar 3. *Indian Phytopathology*, **65**:18-24.

Smith, E.F. 1896. A bacterial disease of the tomato, eggplant and Irish potato. *US Dept. Agric. Div. Veg. Phys. Path. Bull*, **12**:1-26.

Sole, M., Popa, C., Mith, O., Sohn, K. H., Jones, J.D., Deslandes, L. *et al*. 2012. The awr gene family encodes a novel class of *Ralstonia solanacearum* type III effectors displaying virulence and avirulence activities. *Molecular Plant Microbe Interactions,* **25**:941-953.

Stock, J.B. and Surette, M.G. 1996. Chemotaxis, In *Escherichia coli and Salmonella cellular and molecular biology*.1103–1129 *ASM Press, Washington, DC*.

Sun, S., Wu, H. X., and Wang, J. 2013. Research review on the bacterial wilt of Casuarina.*Forest Pest and Disease*, **32**:29–34.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**:2725-2729.

Tans-Kersten, J., Guan, Y. and Allen, C. 1998. *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. *Applied and Environmental Microbiology*, **64**:4918-4923.

Tans-Kersten J., Huang H. and Allen, C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. *Journal of Bacteriology*, **183**:3597-3605.

Thomas, S., Holland, I.B. and Schmitt, L. 2014. The type 1 secretion pathway—the hemolysin system and beyond. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, **1843**:1629-1641.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**:4673-4680.

USDA.2003. Biological control of Fusarium wilt and other soil-borne pathogenic fungi. http://www.ars.usda.gov/research/projects/projects.htm?ACCN_NO=406590&fy=2003.

Ustun, S. and Bornke, F. 2014. Interactions of Xanthomonas type-III effector proteins with the plant ubiquitin and ubiquitin-like pathways. *Frontiers in Plant Science*, **5**:736.

Valls, M., Genin, S. and Boucher, C. 2006. Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*. *PLoS Pathogens*, **2**:e82.

Van Elsas, J. D., Kastelein, P., de Vries, P. M. and Van Overbeek, L. 2001. Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. *Canadian Journal of Microbiology*, **47**: 842-854

Van Gijsegem, F., Vasse, J., Camus, J.C., Marenda, M. and Boucher, C. 2000. *Ralstonia solanacearum* produces Hrp-dependent pili that are required for PopA secretion but not for attachment of bacteria to plant cells. *Molecular Microbiology*, **36**:249-260.

Van Gijsegem, F., Vasse, J., Rycke, R.D., Castello, P. and Boucher, C. 2002. Genetic dissection of the *Ralstonia solanacearum hrp* gene cluster reveals that the HrpV and HrpX proteins are required for Hrp pilus assembly. *Molecular Microbiology*, **44**:935-946.

Vasse, J., Frey, P. and Trigalet, A. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. *Molecular Plant Microbe Interactions*, **8**:241-251.

Vasse, J., Genin, S., Frey, P., Boucher, C. and Brito. B. 2000. The *hrpB* and *hrpG* regulatory genes of *Ralstonia solanacearum* are required for different stages of the tomato root infection process. *Molecular Plant Microbe Interactions*, **13**:259-267.

Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K. and Balandreau, J. 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas] phenazinium,[Pseudomonas*] *pyrrocinia* and [*Pseudomonas] glathei* as *Burkholderia*. *International Journal of Systematic and Evolutionary Microbiology*, **48**:549-563.

Vincze, T., Posfai, J. and Roberts, R. J. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research*, **31**:3688-3691.

Vos, M. and Velicer, G.J. 2006. Genetic population structure of the soil bacterium *Myxococcus xanthus*at the centimeter scale. *Applied and Environmental Microbiology*, **72**:3615-3625.

Wang, J., Zhou, Z., He, F., Ruan, Z., Jiang, Y., Hua, X. and Yu, Y. 2018.The role of the type VI secretion system vgrG gene in the virulence and antimicrobial resistance of *Acinetobacter baumannii* ATCC 19606. *PloS One*, **13**:e0192288.

Weber, B.S., Hennon, S.W., Wright, M.S., Scott, N.E., de Berardinis, V., Foster, L.J., Ayala, J.A., Adams, M.D. and Feldman, M.F. 2016. Genetic dissection of the type VI secretion system in Acinetobacter and identification of a novel peptidoglycan hydrolase, TagX, required for its biogenesis. *MBio*, **7**:01253-16.

Wei, H.L., Zhang, W. and Collmer, A. 2018. Modular Study of the Type III Effector Repertoire in *Pseudomonas syringae* pv. tomato DC3000 Reveals a Matrix of Effector Interplay in Pathogenesis. *Cell reports*. **23***:*1630-1638.

Wicker, E., Grassart, L., Coranson-Beaudu, R., Mian, D., Guilbaud, C. and Prior, P. 2004. Emerging strains of *Ralstonia solanacearum* in Martinique (French West Indies): a case study for epidemiology of bacterial wilt. In *I International Symposium on Tomato Diseases.* **695**:145-152.

Wicker, E., Grassart, L., Coranson-Beaudu, R., Mian, D., Guilbaud, C., Fegan, M. and Prior, P. 2007. *Ralstonia solanacearum* strains from Martinique (French West Indies) exhibiting a new pathogenic potential. *Applied and Environmental Microbiology*, **73**:790-6801.

Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K. and De Ley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as Acidovorax avenae subsp. avenae subsp. nov., comb. nov., Acidovorax avenae subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *International Journal of Systematic and Evolutionary Microbiology*, **42**:107-119.

Wubshet, Z. 2018. Economic importance and management of Ginger Bacterial Wilt caused by *Ralstonia solanacearum.International Journal of Research Studies in Agricultural Sciences*, **2**:1-11.

Wuthiekanun, V. and Peacock, S.J. 2006.Management of melioidosis. *Expert Review of Anti-Infective Therapy*, **4**:445-455.

Xue, Q.Y., Yin, Y.N., Yang, W., Heuer, H., Prior, P., Guo, J.H. and Smalla, K. 2011. Genetic diversity of *Ralstonia solanacearum* strains from China assessed by PCR-

based fingerprints to unravel host plant-and site-dependent distribution patterns. *FEMS Microbiology Ecology*, **75**:507-519.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiology and Immunology*, **36**:1251-1275.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. and Nishiuchi, Y. 1995. Transfer of Two *Burkholderia* and An *Alcaligenes* Species to *Ralstonia* Gen. Nov. Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) Comb. Nov., *Ralstonia solanacearum* (Smith 1896) Comb. Nov. and *Ralstonia eutropha* (Davis 1969) Comb. Nov. *Microbiology and Immunology*, **39**:897-904.

Yao, J. and Allen, C. 2006. Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum*. *Journal of Bacteriology*, **188**:3697-708.

Yu, S.L., Wang, C.T., Yang, Q.L., Zhang, D.X., Zhang, X.Y., Cao, Y.L., Liang, X.Q. and Liao, B.S. 2011. Peanut genetics and breeding in China. *Shanghai Scientific and Technology Press, Shanghai, China*.

Zhang, L., Xu, J., Xu, J., Chen, K., He, L. and Feng, J. 2012. TssM is essential for virulence and required for type VI secretion in *Ralstonia solanacearum*. *Journal of Plant Diseases and Protection*, **119**:125-134.

Zhang, L., Xu, J., Xu, J., Zhang, H., He, L. and Feng, J. 2014. TssB is essential for virulence and required for type VI secretion system in *Ralstonia solanacearum*. *Microbial Pathogenesis*, **74**:1-7.

Zhou, M.Y. and Gomez-Sanchez, C.E. 2000.Universal TA cloning. *Current Issues in Molecular Biology*, **2**:1-8.

Zoued, A., Durand, E., Bebeacua, C., Brunet, Y. R., Douzi, B., Cambillau, C., *et al*. 2013. TssK is a trimeric cytoplasmic protein interacting with components of both phage-like and membrane anchoring complexes of the type VI secretion system. *Journal of Biological Chemistry*, **288**:27031-27041.

Zoued, A., Brunet, Y.R., Durand, E., Aschtgen, M.S., Logger, L., Douzi, B., Journet, L., Cambillau, C. and Cascales, E. 2014.Architecture and assembly of the Type VI secretion system. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, **1843**:1664-1673.

Zoued, A., Durand, E., Brunet, Y.R., Spinelli, S., Douzi, B., Guzzo, M., Flaugnatti, N., Legrand, P., Journet, L., Fronzes, R. and Mignot, T. 2016.Priming and polymerization of a bacterial contractile tail structure. *Nature*, **531**:59-63.

Zumaquero, A., Macho, A.P., Rufian, J.S. and Beuzon, C.R. 2010. Analysis of the role of the type III effector inventory of *Pseudomonas syringae pv. phaseolicola* 1448a in interaction with the plant. *Journal of Bacteriology*, **192**:4474-4488.

CHAPTER IX

Appendix

Appendix I

Virulence assay of *R. solanacearum*

Pectinase Assay

Rs-09-161 was spot inoculated on pectin agar plates and RT for 48 h. To visualize pectinase activity the plates were flooded with 1% CTAB solution and incubated for 30 mins. Pectin degradation was observed as a clear halo around the colony.

Cellulase Assay

Rs-09-161 was spot inoculated on carboxy methyl cellulose agar plates and incubated for 48h. The plate was stained with 0.1% congo red solution for 15 min followed by destaining with 1 M NaCl solution. Degradation of cellulose was observed as yellowish halo on a red background.

Appendix II

Media constituents

BG medium

Peptone	10.0 g
Yeast Extract	1.0 g
Tryptone	1.0 g
Glucose	5.0 g
DW	1000 mL
Agar	15 g

TZC

TZC agar: One mL of 1% tetrazolium chloride was added per liter BG agar to make one litre TZC agar. 1% stock of TZC was prepared by dissolving 1.0 g TZC in 100 mL DW and filter sterilized.

Luria Bertani (LB) medium

Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
DW	1000.0 mL
pH	7.0
Agar	15.0 g

King's B (KB) medium

Peptone	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$.7H2O	1.5 g
Glycerol	10.0 mL
DW	1000.0 mL
Agar	15.0 g

10X strength M63 Media Stock

KH_2PO_4	3.75 g
K_2HPO_4	8.75 g
$(NH_4)_2SO_4$	2.5 g
$FeSO_4$ (1mg/ml)	625 µL
D.W.	500 mL

One fourth strength M63 medium

Flask A	
10X M63	50 mL
Glycerol	2 mL
D.W.	200 mL
Flask B	
Agar	6.69 g
D.W.	250 mL

Mix the flask after autoclaving and add the following components to the hot media

1 M $MgSO_4$	1.25 mL
20% Glucose	12.5 mL

Pectin agar medium

Citrus pectin	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
pH	7.0
Distilled water	1000.0 mL

For pectinase assay, the plates were Flood plates with 1% CTAB solution. Pectolytic colonies show clear halo

Carboxy Methyl Cellulose (CMC) medium

CMC	10.0 g
NaNO3	1.0 g
K2HPO4	1.0 g
KCl	1.0 g
MgSO4. 7H2O	0.5 g
Yeast extract	0.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1000.0 mL

Appendix III

Buffers and Reagents

10 X Phosphate Buffered saline (PBS)	80.0 g of NaCl 2.0 g of KCl 14.4 g of $Na_2HPO_4.2H_2O$ 2.4 g of KH_2PO_4 Make up the volume to 1000 mL.
0.5 M EDTA pH 8.0	146.12 g EDTA 600 mL D.W. Adjust pH to 8.0 with NaOH Make up the volume to 1000 mL.
1 M Tris-HCl pH 8.0	121.1 g of Tris base 600 mL D.W. Adjust pH to 8.0 with HCl Make up the volume to 1000 mL.
5M NaCl	14.6 g of sodium chloride Make up the volume to 50 mL.
CTAB/NaCl solution	2.05 g of NaCl 40 mL D.W. 5.0 g CTAB, dissolve by heating and stirring Make up the volume to 50 mL.
50 X Tris acetate EDTA (TAE) Buffer	242.0 g of Tris Base 57.1 mL of Glacial Acetic acid 100 mL 0.5 M EDTA, pH 8.0 Make up the volume to 1000 mL.

1.5 M Tris-HCl pH 8.8	18.66 g of Tris base 80 mL D.W. Adjust pH to 8.8 with HCl Make up the volume to 100 mL.

1.5 M Tris-HCl pH 6.8	18.66 g of Tris base 80 mL D.W. Adjust pH to 6.8 with HCl Make up the volume to 100 mL.

30% Acrylamide mix	29 g acrylamide 1 g bisacrylamide Make up the volume to 1000 mL Store in amber colored bottle at 4^{o}C.

10% Ammonium per sulfate	One g APS 0.1 g bisacrylamide 1 mL D.W. Prepared freshly

10 X Tris-Glycine buffer	30.3 g Tris base 144 g glycine 10 g SDS Make up the volume to 1000 mL

Transfer buffer	0.60 g Tris base 2.88 g glycine 1 g SDS 20 mL methanol Make up the volume to 1000 mL. The transfer buffer was used within a week of preparation.

Resolving gel (10%)		Stacking gel (6%)	
Reagent	Volume (mL)	Reagent	Volume (mL)
H_2O	4.0	H_2O	5.296
30% Acrylamide mix	3.3	30% Acrylamide mix	2.0
1.5 M Tris-HCl, pH 8.8	2.5	1.5 M Tris-HCl, pH 6.8	2.5
10% SDS	0.1	10% SDS	0.1
10% APS	0.1	10% APS	0.1
TEMED	0.004	TEMED	0.004

Appendix IV

Accession Numbers for virulence factors in *R. solanacearum*: Rs-09-161 and Rs-10-244.

Virulence factor	Gene Name	Gene bank accession numbers	
		Rs-09-161	Rs-10-244
EPS	*epsA*	KY661391	KY661392
	epsB	KY661393	KY661394
	epsC	KY661395	KY661396
	epsE	KY661397	KY661398
	epsF	KY661399	KY661400
	epsP	KY661401	KY661402
	epsR	KY661403	KY661404
PCWDE	*pehA*	KY661405	KY661406
	pehB	KY661407	KY661408
	pehC	KY661409	KY661410
	pme	KY661411	KY661412
	egl	KY661413	KY661414
	cbhA	KY661415	KY661416
Chemotaxis	*cheA*	KY661427	KY661428
	cheW	KY661417	KY661418
Swimming motility	*fliC*	KY661423	KY661424
	flgM	KY661425	KY661426
Twitching motility	*pilA*	KY661419	KY661420
	pilP	KY661421	KY661422

Appendix V

List of strains used in the study

Chapter	Strain	Details
Chapter IV	Ec-hrpB	hrpB::pTZ57R/T
	Ec-hrcV	hrcV::pTZ57R/T
	Ec-TssM	TssM::pTZ57R/T
	Ec-TssH	TssH::pTZ57R/T
	Ec-Hcp	Hcp::pTZ57R/T
	Ec-VgrG3	VgrG3::pTZ57R/T
	Ec-OmpA	OmpA::pTZ57R/T
	Rs-09-161	WT
	Rs-hrpB^{-}	hrpB::pTZ57R/T mutant
	Rs-hrcV^{-}	hrcV::pTZ57R/T mutant
	Rs-TssM^{-}	TssM::pTZ57R/T mutant
	Rs-TssH^{-}	TssH::pTZ57R/T mutant
	Rs-Hcp^{-}	Hcp::pTZ57R/T mutant
	Rs-VgrG3^{-}	VgrG3::pTZ57R/T mutant
	Rs-OmpA^{-}	OmpA::pTZ57R/T mutant
Chapter V	Rs-hrpB^{-}	hrpB::pTZ57R/T mutant
	Rs-hrcV^{-}	hrcV::pTZ57R/T mutant
	Rs-TssM^{-}	TssM::pTZ57R/T mutant
	Rs-TssH^{-}	TssH::pTZ57R/T mutant
	Rs-Hcp^{-}	Hcp::pTZ57R/T mutant
	Rs-VgrG3^{-}	VgrG3::pTZ57R/T mutant
	Rs-OmpA^{-}	OmpA::pTZ57R/T mutant

Chapter	Strain	Details
Chapter VI	S17-1λpir	KmR, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir.
	Ec-RipAM	RipAM::pENTR-SD-D-TOPO
	Ec-RS15E	RS15E::pENTR-SD-D-TOPO
	One Shot® ccdB Survival™ 2 T1R chemically competent cells	ccdB resistant cell for maintanance of pRCG-Pep-GWY vector
	Ec-pRCG-Pep-GWY	pRCG-Pep-GWY
	Ec-pRCG-RipAM	pRCG-Pep-RipAM
	Ec-pRCG-RS15E	pRCG-Pep-RS15E
	Rs-RipAM	pRCG-Pep-RipAM in wild type strain Rs-09-161
	Rs-hrpB-RipAM	pRCG-Pep-RipAM cloned in Rs-hrpB-mutant
	Rs-hrcV-RipAM	pRCG-Pep-RipAM cloned in Rs-hrcV^{-} mutant
	Rs-RS15E	pRCG-Pep-RS15E wild type strain Rs-09-161
	Rs-hrpB-RS15E	pRCG-Pep-RS15E cloned in Rs-hrpB-mutant
	Rs-hrcV-RS15E	pRCG-Pep-RS15E cloned in Rs-hrcV-mutant

CHAPTER X

Publications

Ph.D. publcations

Asolkar, T. and Ramesh, R. 2018. Identification of virulence factors and type III effectors of phylotype I, Indian *Ralstonia solanacearum* strains Rs-09-161 and Rs-10-244. *Journal of Genetics*, **97**:55-66.

Asolkar, T. and Ramesh, R. 2018. Development of T3SS Mutants (*hrpB*$^{-}$ and *hrcV*$^{-}$) of *Ralstonia solanacearum*, Evaluation of Virulence Attenuation in Brinjal and Tomato-A Pre-requisite to Validate T3Es of *R. solanacearum*. *Indian Journal of Microbiology*, **58**: 372-380.

Other Publications

Ramesh, R., Achari, G., **Asolkar, T**., Dsouza, M. and Singh, N.P. 2016. Management of bacterial wilt of brinjal using wild brinjal (*Solanum torvum* Sw) as root stock. *Indian Phytopathology*, **69**:260-265.

Ramesh, R., Gaitonde, S., Achari, G., **Asolkar, T**., Singh, N.P., Carrere, S., Genin, S. and Peeters, N. 2014. Genome sequencing of *Ralstonia solanacearum* biovar 3, phylotype I, strains Rs-09-161 and Rs-10-244, isolated from eggplant and chili in India. *Genome Announcements*, **2**:e00323-14.

Poster Presentation: Secured 1st place for poster presentation

Asolkar, T. and Ramesh, R. 2016. Molecular characterisation and virulence assays of *Ralstonia solanacearum*, a bacterial wilt causing phytopathogen.In abstract of "National Seminar on Advances in Life Sciences" PP-27 held on 7th-8th December 2015 at St. Xavier's College, Mapusa.