“*Ralstonia solanacearum* in eggplant: Diversity, virulence and factors influencing its survival in soil’’

A Thesis submitted to Goa University for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

By

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Work carried out under the guidance of

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Statement

I hereby declare that the thesis entitled **"*Ralstonia solanacearum* in eggplant: Diversity, virulence and factors influencing its survival in soil"** submitted to Goa University, for the award of the degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY** is a record of original and independent work carried out by me during October 2008 - June 2014, in the **Plant Pathology Laboratory, ICAR, Research Centre for Goa, Old-Goa, Goa** under the supervision of **Dr. R. Ramesh,** (Sr. Scientist, Plant pathology), ICAR, Research Complex for Goa, Old Goa, Goa and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

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

Signature of the Student

Sapna Suhas Gaitonde

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Dedicated

to

my

beloved

father

Abbreviations

$(NH_4)_2SO_4$	Ammonium sulphate
ANOVA	Analysis of Variance
BMM	Boucher's minimal medium
BW	Bacterial wilt
$CaCl_2$	Calcium chloride
CAS	Chrome azurol S
CFU	Colony Forming Units
CMC	Carboxymethyl cellulose
CTAB	cetyltrimethylammonium bromide
CWDE	Cell wall degrading enzymes
DAI	Days after inoculation
DMSO	Dimethyl sulfoxide
DNA	Dioxy ribonucleic acid
DNSA	Dinitro salicylic acid
dNTP	Deoxyribonucleotide triphosphate
EC	Electrical conductivity
EDTA	Ethylene diamine tetra acetic acid
EG	Endoglucanase
EPS	ExtRacellular polysaccharide
EPS	Exopolysaccharide
EtBr	Ethidium bromide
$FeCl_3$	Ferric Chloride
$FeSO_4$	Ferrous sulphate
Fig.	Figure
FYM	Farm yard manure
GC-MS	Gas chromatography Mass Spectroscopy

Abbreviations

GLM	General linear Model
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
K_2HPO_4	Dipotassium hydrogen phosphate
KBr	Potassium bromide
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
$MgSO_4$	Magnesium sulphate
MLSA	Multilocus sequence analysis
MLST	Multi Locus Sequence typing
Na_2HPO_4	Disodium hydrogen phosphate
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate
Na_2SO_3	Sodium thiosulphate
$NaH_2PO_4.H_2O$	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NJ	Neighbour Joining
O.D.	Optical density
PAME	3-hydroxypalmitic acid methyl ester
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PG	Polygalacturanase
PGA	Polygalacturonic acid

Abbreviations

rep-PCR	Repetative Polymerase chain reaction
rRNA	Ribosomal Ribonucleic acid
RT	Room Temperature
SDS	Sodium dodecyl sulphate
sp.	Species
T2SS	Type II Secretion system
T3SS	Type III Secretion system
TAE	Tris Acetate EDTA
TE	Tris EDTA
TMPD	Tetramethyl paraphynylene diamine
Tukey's HSD	Tukey's honest significant difference
TZC	Triphenyl tetrazolium chloride
UV	Ultraviolet
ZOD	Zone of degradation

Units of measurements

°C	Degree celcius
CFU	Colony Forming Units
g	Gram
ha	Hectar
h	Hour
kg	Kilogram
L	Litre
µg	Microgram
µm	Micrometer
µ	Micron
mg	Milligram
mL	Millilitre
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
ng	Nanogram
pmol	Picomoles
rpm	Revolution per minute
sec	Seconds
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume

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Introduction

Ralstonia solanacearum is a soil-borne beta-proteobacterium that causes bacterial wilt in 450 plant species of 54 families across the countries. *R. solanacearum* is a highly heterogeneous species and contains isolates from all geographical regions. All *R. solanacearum* strains have traditionally been classified into races (1-5) based on host range and biovars (six) based on the ability to acidify carbohydrate substrates. Recently, the phenotypic and genotypic variation of the species was further classified into four phylotypes based on the sequences of selected genes and this correlates the strains with their geographical origin. Genome of *R. solanacearum* genome is organized as chromosome and megaplasmid. Till date, the genome sequences of 13 *R. solanacearum* strains are publicly available.

R. solanacearum infects plants through roots and spreads rapidly in the xylem vessels and suppresses plant defense mechanisms via the type III secretion system. In the xylem vessels, the bacteria multiply extensively and produce large amounts of exopolysaccharide ensuing collapse of the water flow causing the wilting symptoms and eventually plant death.

R. solanacearum possesses a wide array of virulence factors. This bacterium survives in soil in the absence of the host and also in decaying plant debris, contaminated water, rhizosphere of non-host plants and asymptomatic weeds.

In Goa, solanaceous vegetables particularly eggplant are cultivated throughout the year. Eggplant cultivation is severely affected due to bacterial wilt and the disease incidence ranges from 30-100%. *Agassaim* is the popular and high yielding local cultivar of Goa which is highly susceptible to bacterial wilt.

Researchers are trying to combat this bacterial wilt pathogen through various physical, cultural, chemical and biological methods. Bacterial wilt could effectively be managed through crop rotation, host resistance and chemical control to a certain extent. In spite of various efforts, it is very difficult to control bacterial wilt using a single strategy as the pathogen is soil borne and has a broad host range in addition to the existence of vast genetic variation. Further, breakdown of host resistance is reported regularly. Therefore studying this pathogen is very important for making appropriate decision on disease management. Hence I have started my programme with the following objectives.

- To study the diversity of *Ralstonia solanacearum* infecting eggplant from Goa and adjoining regions and its characterization.
- To study the pathogenicity and virulence factors of *R. solanacearum* infecting eggplant.
- To study the environmental and soil factors influencing the *R. solanacearum* infection.
- To study the effects of botanicals on the inhibition of *R. solanacearum*.

The overall goal of this thesis include unraveling the phenotypic and genotypic characteristics of *R. solanacearum* isolates from Goa, studying the genetic diversity of the pathogen, studying its pathogenic and virulence factors, understanding the environment and soil factors that are responsible for its survival and dissemination and exploring the plant products for the pathogen inhibition.

Chapter I cover the exhaustive review of work done both national and international level on the selected topic. Chapter II is written on isolation of the pathogen from different

geographical regions and its characterization using various techniques. Chapter III deals with the study on pathogenicity and virulence factors of *R. solanacearum* infecting eggplant. Chapter IV reports the study of various environmental and soil factors in the survival and multiplication of the pathogen. Chapter V elaborates the possibility of using plant extracts to inhibit the growth of pathogen and further characterization of the inhibitory compounds.

CHAPTER I

Review of literature

1.1. Introduction

Ralstonia solanacearum is a soil-borne bacterium which is the causal agent of Bacterial Wilt (BW) disease in about 450 plant species of 54 families from both monocots and dicots (Wicker *et al.*, 2007) and the most susceptible hosts are potatoes, tomatoes, pepper, eggplant, and groundnut. In a recent survey, it is found that *R. solanacearum* ranked second in the list of the most important bacterial pathogens, based on scientific and economic importance, only after *Pseudomonas syringae* (Mansfield *et al*., 2012). In tropical and sub-tropical regions, BW has been the second most devastating disease in potato (CGIAR, 2005). Many economically important crops like potato, tomato, eggplant, cooking banana and peanut are damaged due to this disease (Aggarwal *et al*., 2006; Biswas and Singh, 2007; Sharma and Kumar, 2009; Remenant *et al.*, 2010). The bacterium belongs to the β subdivision of the proteobacteria class, order Burkholderiales and family Ralstoniaceae.

BW is known to be soil-borne and seed-borne disease in potato as it disseminates via infected tubers (Moslemkhany *et al.*, 2005). It was reported by Lemay *et al.* (2003) that *R. solanacaerum* infection is not localized as it moves from one host to the other in the soil. Ramesh *et al.* (2011a) further reported that *R. solanacearum* infection could spread through contaminated water and weeds in the Solanaceae family. This pathogen can lie dormant in water or soil until it enters the roots of a host and colonizes its water-conducting vessels, from where it spreads throughout the plant and multiplies to a high

population density. The 19th century reports have indicated the presence of *R. solanacearum* infection in Asia, South America, USA and Australia (Kelman, 1953).

1.2. Taxonomy

BW pathogen was first named as *Bacillus solanacearum.* Later on it was given the synonym *Pseudomonas solanacearum* (Smith, 1896). Further studies on the taxonomy of certain non-fluorescent *Pseudomonas* species led to creation of new genus *Burkholderia* to encompass the dissimilarities found in this group (Yabuuchi *et al.*, 1992). Further investigations proved that *Burkholderia solanacearum* was thoroughly separated from the rest of *Burkholderia.* For nearly a century, *R. solanacearum* was known to be a member of the *Pseudomonas* rRNA homology group II (*P. mallei*, *P. caryophylli*, *P. cepacia*, *P. pickettii*, and *P. gladioli*) and was called *P. solanacearum* (Palleroni and Doudoroff, 1971). Consequently new genus *Ralstonia* was designated and *Burkholderia solanacearum* was renamed as *R. solanaceraum* (Yabuuchi *et al.*, 1995). Based on rRNA homology group II (nonfluorescent) of the taxon *Pseudomonas* (Poussier *et al.*, 2000), Denny *et al*., (2001) further established the genus *Ralstonia* based on the 16SrRNA genes sequences and the chemotaxonomic data. Since then, the bacterium is named *R. solanaceraum*. Bacteria that are taxonomically related and often misidentified as *R. solanaceraum* include *Pseudomonas syzigii*, the causal agent of Sumartra disease of cloves (*Zingiber aromaticum*) in Java and Sumatra, and *Pseudomonas celebensis*, the blood disease bacterium (BDB) of Musaceae in Indonesia (Hayward, 2000; Taghavi *et al.*, 1996).

The Table 1.1 below depicts the chronology of name changes of *R. solanaceraum*:

Table 1.1 Chronology of names of *R. solanacearum*

Designated name	**Reference**
Bacillus solanacearum	Smith (1896)
Pseudomonas solanacearum (Smith)	Smith (1914)
Burkholderia solanacearum	Yabuuchi *et al.*, 1992
R. solanaceraum	Yabuuchi *et al.*, 1995

At present the bacterium is classified under

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Burkholderiales

Family: Ralstoniaceae (CABI, 2013)

1.3. History of BW research

BW disease was first reported from Asia and South America in late 1880's. Smith described the causal organism of BW in 1896. Kelman and Luis Sequeira are among the pioneer scientists, who worked on diverse aspects of the pathogen biology and therefore Kelman (1953; 1954) summarized the research on this disease up to the early 1950’s. He isolated *R. solanaceraum* type strain K60 from a wilted plant from his backyard in North Carolina. Buddenhagan (1986) was another pioneer working for the management of BW (affecting banana and plantains) in Central America described BW as a highly complex disease. During 1970s, research was shifted towards genetics of bacteria and the pathogen diversity was further explored through its broad host range, variable phenotypic and genetic characteristics (Buddenhagen and Kelman, 1964; Cook *et al.*, 1989; Prior and Fegan, 2005). Boucher and his team developed mutagenesis protocol for *R. solanaceraum* and were the first to work on *hrpB* mutants (*hrp* gene cluster required for HR response on non-host). Since then many researchers have worked to unravel many mysteries of virulence mechanism in *R. solanacearum* (Schell, 2000; Denny, 2005). The first complete DNA sequence of type strain GMI1000 has also been completed which provides detailed view of its genome functions (Salanoubat *et al*., 2002).

1.4. Phenotypic characteristics of *R. solanacearum*

R. solanacearum is a Gram-negative rod shaped bacterium, and measures 0.5-0.7 x 1.5-2.0 mm and motile by 1-4 polar flagella. It is aerobic and gives positive results for catalase, oxidase and nitrate reduction. The organism is negative for levan production, starch hydrolysis, indole production and hydrogen sulfide hydrolysis (Denny and

Hayward, 2001). It is non-fluorescent on King's medium B, accumulates poly-B-hydroxybutyrate, and the G+C content of its genomic DNA 66.5-68%. In many studies, it was observed that the optimal growth temperature for most of the strains of this bacterium falls between 28-32°C (Hayward, 1964; Schaad *et al.*, 2001).

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), since 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*. Bacterial colonies grow faster (48-72h) in TZC medium and hence preferred for routine culturing. On solid Casamino acid peptone glucose (CPG) agar medium, the bacterial growth is observed after 36 to 48 h. On addition of tetrazolium chloride (TZC) to CPG medium the bacteria appears white, fluidal, irregular shape with pink centres (Fig. 1.1).

Fig. 1.1 Colony characterisitics of *R. solanacearum* on TZC medium

On further incubation of the colonies turn blood red whorls appear in the centre. Kelman (1954) observed that the avirulent colonies appear as small round, and are dark red.White fluidal colonies with a pink center are normally virulent whereas red, round and convex colonies are typically avirulent. On solid agar medium the colonies appear smooth, shiny and opalscent initially, but become brown with age (Lelliott and Stead, 1987).

1.5. Maintenance and storage conditions of *R. solanacearum* culture

This pathogen easily loses virulence and viability when repeatedly transferred on agar plates and stored at 4°C for longer time (Stead, 1993). Therefore, the culture of *R. solanacearum* can be stored in freeze dried condition or on beads at -80°C. Currier and Morgan (1981) have suggested that *R. solanacearum* cell suspension culture in distilled water can be stored at room temperature without losing their virulence and viability. Marina and El Nashaar (1993) stored bacterial suspension of strains of *R. solanacearum* in sterile distilled water in screw capped tubes. Black and Sweetmore (1993) reported the storage of *R. solanacearum* in distilled water with backup in 20% glycerol and kept at -80 °C. Bringel *et al.* (2003) preserved 50 isolates of *R. solanacearum* from potato and eggplant and evaluated the isolates for any variation in biochemical characteristics, pH, carbon and pathogenicity. According to Grey and Steck (2001) the bacterial cells becomes virulent when applied to soil in the vicinity of plants roots. The techniques most frequently used for preservation are distilled water storage (Kelman and Person, 1961) and lyophilization.

1.6. Host Range

The host range of the bacterium is unusually broad together with hundreds of plant species (Hayward, 1991). *R. solanacearum* infects 29 natural hosts other than potato and tomato (Pradhanang *et al*., 2000). The major hosts of *R. solanacearum* worldwide are given in Table 1.2.

Table 1.2 List of major hosts of *R. solanacearum*

Hosts	References
Lycopersicon esculentum (tomato), *Solanum tuberosum* (potato)	Sequeira, 1998, Davis *et al.*, 2000; Lopes *et al.*, 2005
Capsicum annum (sweet pepper), *Solanum melongena* (eggplant)	
Nicotiana tabacum (tobacco), *Arachis hypogaea* (groundnut)	
*Pelargonium hortorum (*geranium)	Swanson, 2007
Arabidopsis thaliana	Norman *et al.*, 2009, Deslandes *et al.*, 1998
Bananas and *Heliconia spp*	EPPO, 1999
Sunflower	Elphinstone, 2005
Pepper spp. and *Morus spp*	Aragaki and Quinon, 1965; French *et al.*, 1995
Anacardium occidentale (cashew)	Shiomi *et al.*, 1989
Annona spp. (custard apple)	Mayers and Hutton, 1987
Archontophoenix alexandrae (Alexandra palm)	Akiew and Hams, 1990
Artichokes	Aly and El ghafar, 2000
S. nigrum (blacknightshade), *Melampodium perfoliatum*, *Datura stramonium*, *Portulaca oleracea*	NPAG, 2001
Strawberry (Japan)	Goto *et al*., 1978
Cerastium glomeratum, *Drymaria cordata*, *Polygonum capitatum and Stellaria media*	Pradhanang *et al.*, 2000
Solanum dulcamara (bittersweet)	Elphinstone *et al*., 1998
Brassica spp., *Chenopodium album* and *Tropaeolum majus*	Janse *et al.*, 2002
Urtica dioica	Wenneker *et al.*, 1999
Ipomoea batatas (China)	He *et al*., 1983
Eucalyptus (Brazil, China)	Dianese *et al*., 1990
Cassava (Indonesia)	Nishiyama *et al*., 1980
Peanut (China)	Middleton and Hayward, 1990

Kumar *et al*. (2004) reported that Ginger, a monocot was infected with bacterial wilt pathogen in India. Some minor hosts reported include, *Anthurium* spp., cotton (*Gossypium hirsutum*), rubber (*Hevea brasiliensis*), cassava (*Manihot esculenta*), castor beans (*Ricinus communis*), *Pogo stemonpatchouli*, pumpkin, sesame, turmeric. In

addition, many weeds are also hosts of the pathogen and therefore increase the potential of *R. solanacearum* to build up inoculum (Kelman, 1953; Bradbury, 1986; Elphinstone, 2005). From this list, it is apparent that the *R. solanacearum*'s host range is not restricted to solanaceous plants but encompasses many other botanical families among dicots and monocots.

1.7. Geographical Distribution

BW is one of the most important diseases in tropical, subtropical, warm temperate regions and cool temperate areas (Poussier *et al*., 1999). The disease is vastly distributed across the world (Table 1.3).

Table 1.3 Geographical distribution of *R. solanacearum* worldwide

Country	Regions	Crop	Reference
South America	Bolivia, Brazil, Argentina, Chile, Colombia, Uruguay and Peru	Potato tomato	Fegan and Prior, 2005; Davis *et al*., 2000; Lopes *et al*., 2005
North America	Georgia, Arkansas, Alabama and Hawaii Carolina states	Tomato, tobacco and ornamentals	Fegan and Prior, 2005
Central America and Caribbean	Costa Rica, Guadeloupe and Mexico	Banana and wild *Heliconia sp.*	Fegan and Prior, 2005; EPPO, 1999
Europe		Potato	EPPO, 1998

Country	Regions	Crop	Reference
Africa	Burundi, Egypt, Kenya, Libya, Reunion, South Africa, and Zambia, Ethiopia, Rwanda, South Africa, Tanzania and Uganda	Potato, tomato, pepper and tobacco.	Elphinestone *et al.*, 2005 EPPO, 1999; Garcia *et al.*, 1999
Asia and Middle East	China, Japan, India, , Indonesia, Pakistan, Malaysia, Sri Lanka Thailand, Vietnam, Philippines, Lebonan and Iran	tomato, brinjal, sweet pepper and hot peppers	CABI and EPPO, 1998; He *et al.*, 1983; Geddes, 1989; Burney, 1995, Horita and Tsuchiya, 2001

It is suspected that Race 2 biovar 1 might be present in Florida. Biovar 3 strains have been found to decline ginger yields in Hawaii (Yu *et al.*, 2003). Recently, Race 3 strains (biovar 2) have also been established in North America through imported geraniums (*Pelargonium hortorum*) (Swanson *et al.*, 2005). Until now there are no reports for the presence of *R. solanacearum* in Canada (EPPO, 1999; Elphinestone *et al.*, 2005). The disease has also been reported to affect banana production in India, Malaysia, Sri Lanka, Thailand and Vietnam (CABI/EPPO, 1999). Throughout Philippines, Moko and bugtok diseases (affecting banana and plantain) are wide spread (Sequeira, 1998). Strains belonging to biovar 1 (Race 2), Banana Blood Disease pathogen (phylotype 4) are wide spread in Indonesia (Davis *et al.*, 2000); Race 2 infects banana and others crops from

Musaceae; Race 3 infects potato and other crops from solanaceae (Elphinstone, 2005); Race 4 is known to infect ginger (Kumar *et al*., 2004) and Race 5 infects Mulberry and Morus (OEPP/EPPO, 2004). Begum (2005) isolated the pathogen from seeds as well as plant samples obtained from different tomato growing areas of Punjab province (Pakistan). Biovar 3 was reported as pathogenic on eucalyptus (Fouche *et al*., 2006)

Bacterial wilt affects mainly the solanaceous vegetables in India (Singh *et al.*, 1997) and its incidence ranges from 30-100% in Goa on eggplant cultivation (Ramesh, 2006). Its prevalent in Assam, Arunachal Pradesh, Gujarat, Western Maharashtra, Karnataka, Kerala, Madhya Pradesh,, Manipur, Orissa, West Bengal, Meghalya, , Tripura, Mizoram, Andaman and Nicobar Islands (Ramesh *et al.*, 2014b; Sagar *et al.*, 2014; Chandrashekhara *et al.*, 2012; Prasannakumar *et al.*, 2012; Bhattacharya *et al.*, 2003; Shekhawat *et al.*, 1978). Khan *et al.* (1974) reported Asiaticum group of *R. solanacearum* from India on the basis of comparative studies of the isolate infecting solanaceous crops.

1.8. *R. solanacearum species* complex

R. solanacearum is a species complex comprising of closely related isolates that represent more than one species (Fegan and Prior, 2005). These strains were further grouped based on genetic similarities of 16S-23S ITS, *hrpB* and *egl* sequences by Fegan and Prior (2005) and arranged in a phylogenetic tree (Fig. 1.2). This is useful as the isolates can be genetically classified based on 16 S rDNA sequences. DNA-DNA hybridization analysis has proved that very high degree of diversity amongst the strains (Remenant *et al.*, 2011).

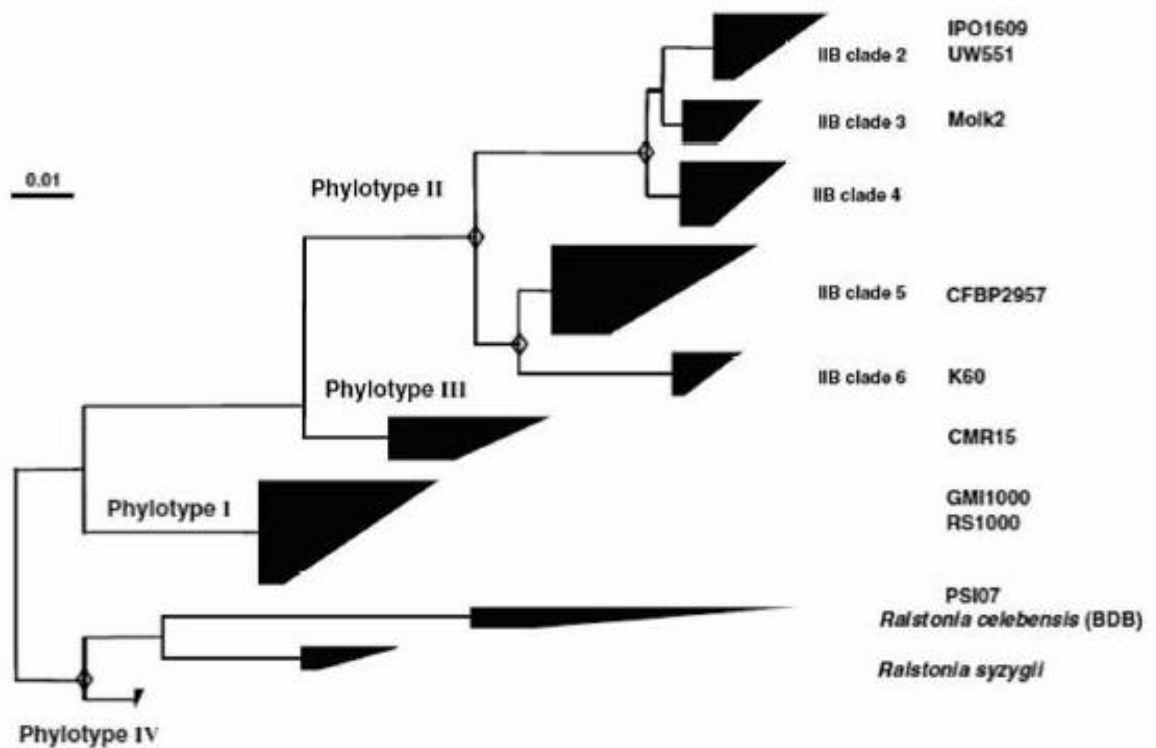


Fig. 1.2 Hierarchical classification scheme showing relationships between *R. solanacearum* species complex based on *egl* gene sequences (Adapted from Fegan and Prior, 2005; Genin, 2010).

Taxonomists and experts have assessed the differences amongst different strains in the species complex depending upon Average Nucleotide Identity (ANI) values. The ANI values over 95% have been considered equivalent to 70% identity as observed in the DNA-DNA hybridization analysis (Remenant *et al.*, 2011; 2010) and hence could provide a confidence threshold for a new classification system of the species (Genin and Denny, 2012). Remanent *et al*. (2011) proposed two new species and 3 sub species and Wicker *et al.* (2012) defined the respective clades (Fig. 1.3). Further, it was confirmed that the species complex is polyphyletic as reported earlier by Vaneechoutte *et al.* (2004).

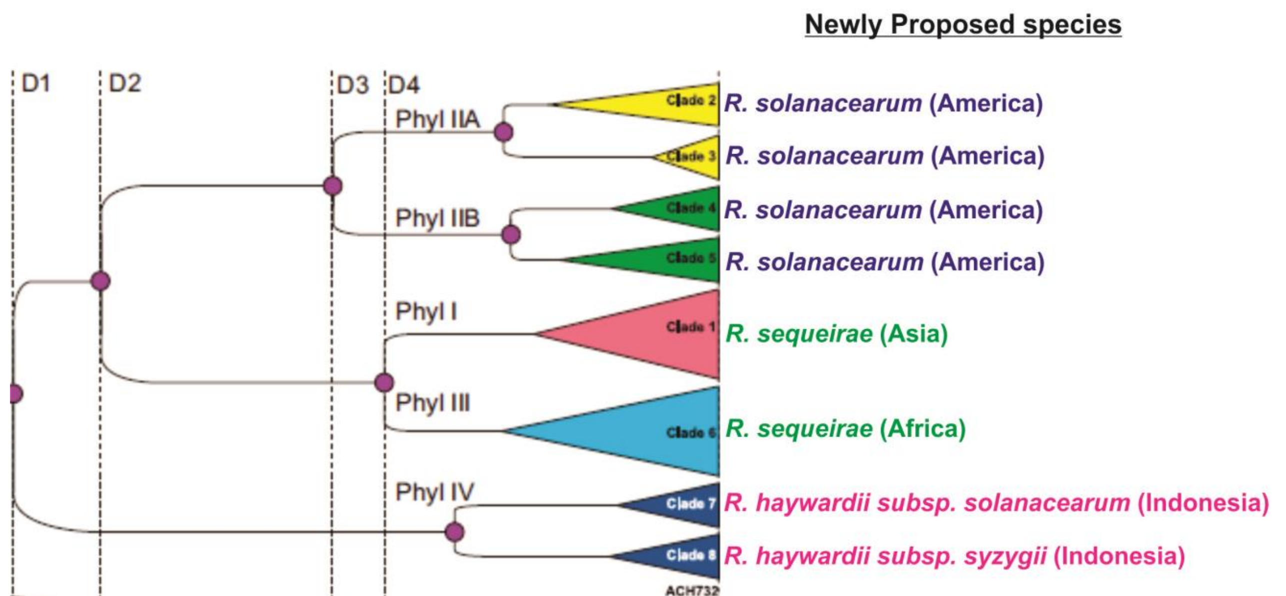


Fig. 1.3 Schematic representation of newly proposed nomenclature and clades within the *R. solanacearum* species complex and their respective geographical location; D-Division; Phyl-Phylotype; Clades (1-8). (Adapted from Remenant *et al.*, 2011; Genin and Denny, 2012; Wicker *et al.*, 2012)

1.8.1. Race determination in *R. solanacearum*

Although *R. solanacearum* is known to have broader host range, different races may exhibit limited host ranges. Klement (1964) developed an injection-infiltration method as a rapid test of pathogenicity of phytopathogenic pseudomonads wherein tobacco plants were used and 10^7 bacterial cells mL^{-1} of bacterial suspension was injected using fine hypodermic needle into the intercellular spaces of the tobacco leaf. Based on Hypersensitive response (HR), the isolates were grouped into races. They were also considered as a group of pathogens that infects a given set of plant varieties. Lozano and Sequeira (1970) developed a leaf infiltration technique on tobacco to differentiate three races of *P. solanacearum* based on HR on plant. Table 1.4 gives the details of the races and the hosts of *R. solanacearum*

Table 1.4 Details of the races and the corresponding hosts of *R. solanacearum*

Race	**Host Range**
1	Tobacco, tomato, diploid bananas eggplant, groundnut, potato, pepper, ginger,, olive, strawberry, geranium, Eucalyptus and many weeds
2	Triploid bananas (AAA, AAB, ABB genotypes), *Heliconia sp.*, Plantains
3	Potato, tomato, some other Solanaceae, Geranium; *Pusa*, *S. dulcamara*, *S. nigrum*, *S. Cinereum*, *Pelargonium spp.*, solanaceous and non solanaceous herbaceous weeds,
4	Ginger
5	Mulberry (*Morus spp.)*

1.8.2. Biovar determination of *R. solanacearum*

Hayward (1964) classified *R. solanacearum* strains into six biovars (Xue *et al.*, 2011) based on their ability to utilize carbohydrates (mannitol, dulcitol, sorbitol, lactose, maltose and cellobiose) (Table 1.5). .

Table 1.5 Tests for biovar determination in *R. solanaceraum* (Hayward, 1964; EPPO, 2004).

Utilization of:	Biovar 1	Biovar 2	Biovar 3	Biovar 4	Biovar 5	Biovar 6
Maltose	-	+	+	-	+	+
Lactose	-	+	+	-	+	+
Cellobiose	-	+	+	-	+	+
Mannitol	-	-	+	+	+	+
Sorbitol	-	-	+	+	-	+
Dulcitol	-	-	+	+	-	-

It has been proved that biovar type appears to be relatively stable in culture. *R. solanacearum* are classified at the intrasubspecific level (Hayward, 1991). Mostly Race 3 is correlated with biovar 2 phenotype (Buddenhagen and Kelman, 1964; Hayward, 1991). Race 1 strains have a broader host range than Race 2 and 3 strains; therefore, these classification systems show a little correlation.

1.8.3. Divisions based on RFLP

Based on Restriction fragment length polymorphism (RFLP) analysis *R. solanacearum* has been classified into 2 divisions' viz. the 'Americanum' division (biovar 1, 2 and N2 strains) and the "Asiaticum" division (biovars 3, 4 and 5 strains). The sequence analysis of the 16SrRNA gene, the 18S-23S rRNA gene intergenic spacer region, the polygalacturonase gene and the endoglucanase gene has confirmed these two divisions.

Furthermore, distinctive genotypic patterns (called Multilocus genotypes) were identified and follow up studies later on led to a further classification that comprised of isolates from Indonesia (Horita *et al*., 2001; Fegan *et al*., 1998., Cook *et al.*, 1989).

1.8.4. Phylogenetic classification

Prior and Fegan (2005) introduced a novel classification scheme for *R. solanacearum* that defines phylotypes. A phylotype can be defined as a monophyletic cluster of strains based on sequence data from the ITS region, *hrpB* gene, and endoglucanase gene. The strains are clustered based on the sequences of four marker genes, i.e. the 16S-23S rRNA gene intergenic spacer (ITS) region, *mut*S, *egl* and *hrp*B sequences (Fegan *et al*., 1998; Poussier *et al*., 2000; Fegan and Prior, 2005). This scheme helps in correlating the strains with the geographical origin. Fegan and Prior (2005) determined a single multiplex PCR reaction based on the 16S-23S ITS region

Phylotype I strains originate from Asia, phylotype II from America, Phylotype III from Africa, and phylotype IV from Indonesia and Australia (Prior and Fegan 2005). Castillo and Greenberg (2007) confirmed the robustness of the phylotyping scheme by, applying multilocus sequence typing (MLST) on 58 strains representing the four phylotypes and further subdivided phylotype II into two subgroups, denoted IIa and IIb. Although, Sagar *et al.* (2014) has reported that the Indian potato isolates of *R. solanacearum* belong to three phylotypes (I, II, and IV), no other phylotype of *R. solanacearum* was determined infecting solanaceous vegetables. Xu *et al.* (2009) reported that majority of isolates belonged to the Asian phylotype I and biovars 3 and 4.

1.8.5. Sequevar determination

A single phylotype consists of sequevars that have a highly conserved sequence within the target locus. RFLP MLGs are known to produce the same or similar groups of strains comparable to sequevars. Over 50 sequevars were differentiated based upon the sequence of endoglucanase gene (Xu *et al.*, 2009). It was further demonstrated that sub groups within each sequevar termed as clonal lines could also be defined using various genomic fingerprinting techniques. Techniques used to determine phylogeny of *R. solanacearum*: Total genomic fingerprinting, PCR-RFLP, AFLP, (rep)-PCR and tRNA-anchored PCR.

1.9. Genetic characterization of *R. solanacearum*

In order to devise successful breeding and integrated management programme a detailed study of the pathogen diversity is a must (Sanchez Perez *et al*., 2008). Various techniques were employed to investigate the phylogeny of *R. solanacearum* (Table 1.6). Genomic fingerprinting studies by Smith *et al*., (1995) and Frey *et al*., (1996) have specifically addressed these aspects by genomic fingerprinting: macro restriction of genomic DNA resolved by pulsed-field gel electrophoresis (MR-PFGE) and PCR with prime sets to conserved bacterial repetitive DNA motifs (rep-PCR). The DNA-based methods such as RFLP, 16S rDNA sequence based polymerase chain reaction (rep-PCR), Pulsed field gel electrophoresis (PFGE) etc. are effective in determining the diversity and genetic relationship within *R. solanacearum* strains (Horita and Tsuchiya, 2001; Dookun *et al*., 2001; Smith *et al*., 1995; Poussier *et al*., 1999; Horita *et al*., 2005; Seal *et al*., 1999; Lee *et al*., 2001; Peters *et al*., 2004).

Table 1.6 Various DNA based methods for genetic characterization of *R. solanacearum* isolates

No.	Technique	References
1	Total genomic fingerprinting	Gillings and Fahy, 1993; Van der Wolf *et al*., 1998
2	PCR-RFLP	Xue *et al*., 2010; Fouche weich *et al*., 2006; Villa *et al*., 2003; Poussier *et al*., 2000b; Poussier *et al*., 1999; Gillings *et al*., 1993
3	Rep-PCR	Stevens and Elsas, 2010; Horita *et al*., 2010; Xue *et al*., 2010; Norman *et al*., 2009; Nouri *et al*., 2009; Khakvar *et al*., 2008; Ivey *et al*., 2007; Kumar *et al*., 2004; Janse *et al*., 2004; Horita and Tsuchiya, 2001; Horita and Tsuchiya, 2000; Dookun *et al*., 2001; Robertson *et al*., 2001;

		References
		Thwaites *et al.*, 1999; Jaunet and Wang, 1999; Van der Wolf et *al.*, 1998; Frey *et al.*, 1996; Smith *et al.*, 1995
4	RAPD	Mesquita *et al.*, 1998; Grover *et al.*, 2006; Mazurier *et al.*, 1992; Parent *et al.*, 1996; Hilton and Penn, 1998; Khoodoo *et al.*, 2002; Chen *et al.*, 2003; He *et al.*, 2003; Jaunet *et al.*, 1996; Jaunet and Wang, 1999
5	PFGE	Ramesh *et al.*, 2011; Hong *et al.*, 2008; Smith *et al.*, 1995; Khakvar *et al.*, 2008; Stevens and Elsas, 2010; Frey *et al.*, 1996
6	AFLP	Yu *et al.*, 2003; Poussier *et al.*, 2000; Van der Wolf *et al.*, 1998; Jeong *et al.*, 2007
7	16S sequencing	Stevens and Elsas, 2010; Jeong *et al.*, 2007; Villa *et al.*, 2005; Poussier *et al.*, 2000
8	Gene sequencing	Cellier and Prior, 2010; Xue *et al.*, 2010; Toukam *et al.*, 2009; Xu *et al.*, 2009; Xu *et al.*, 2009; Liu *et al.*, 2009; Hong *et al.*, 2008; Ji *et al.*, 2007; Wicker *et al.*, 2007; Villa *et al.*, 2005; Poussier *et al.*, 1999
9	tRNA-anchored PCR	Seal *et al.*, 1992

PCR-RFLP- Polymerase chain reaction-Restriction fragment length polymorphism

Rep-PCR- Repetitive Extragenic Palindromic polymerase chain reaction

RAPD- Randomly amplified polymorphic DNA

PFGE-Pulse field gel electrophoresis

AFLP- Amplified Fragment Length Polymorphism

1.9.1. PCR-RFLP

Kumar *et al.* (2004) employed PCR-RFLP-PCR technique to genetically analyse extremely pathogenic isolate from ginger and other hosts (India) and clustered them at 100% similarity coefficient with their host origin and biovar. The PCR-RFLP of *R. solanacearum* strains from Australia using Rs759/760 primers (Opina *et al*., 1997) has been studied and has proved to be effective for prompt identification of race and biovar of the pathogen (Roncal *et al*., 1999). Thammakijjawat *et al.* (2004) used PCR-RFLP of 280 bp fragment restricted with *Hae* III and *Msp* I and successfully differentiated the biovar 2 Race 3 *R. solanacearum* from biovars 3, 4 and N2. Gillings *et al*. (1993) could divide *R. solanacearum* into 6 groups after digestion of PCR products with *Hae* III. The 282-bp fragment in *R. solanacearum* on RFLP analysis could divide the strains from Asia into three groups (Villa *et al.*, 2003).

1.9.2. RAPD

RAPD markers were used of by many researchers for strain differentiation (Mesquita *et al*., 1998), identification and genetic differentiation of a number of bacteria (Akopyanz *et al*., 1992; Mazurier *et al*., 1992; Parent *et al*., 1996; Hilton and Penn, 1998; Khoodoo *et al*., 2002) and for biovar distinction revealing their origin (Silveira *et al*., 2005) Grover *et al*. (2006) studied genetic diversity amongst 44 field isolates and 22 *in vitro* generated clones of *R. solanaceraum* using RAPD and observed high degree of variability within the *R. solanaceraum* isolates. This method is also employed in different regions of Taiwan (Jaunet *et al*., 1996; Jaunet and Wang, 1999) and in China (Chen *et al*., 2003) for determining the genetic diversity among *R. solanacearum* isolates.

1.9.3. Rep-PCR

Repetitive element palindromic polymerase chain reaction (Rep-PCR) is known to be the most useful DNA fingerprint method for determining the genetic relationships within the *R. solanacearum* isolates. Isolates of *R. solanacearum* are genetically characterized by (rep-PCR) using ERIC, REP and BOX primers by many researchers. Horita *et al*. (2005) reported that each primer set (REP, ERIC and BOX) gave unique fingerprint patterns among biovar N2 strains which differed in geographical origin. There was no correlation derived from Rep-PCR clusters that were observed between the biovar type and the geographical origin of the *R. solanacearum* strains isolated from tomato as reported by Januet and Wang (1999). However it was found that highly pathogenic isolates of ginger from India were clustered together with 100% similarity coefficient (Kumar *et al*., 2004). Xue *et al.* (2011) used 75% similarity coefficient to define BOX and ERIC fingerprints. Frey *et al.* (1996) reported that ERIC and Rep-PCR profiles were useful in differentiating biovar 1 and 3 strains of Race 1. Smith *et al.* (1995) distinguished a Race 3 biovar N2 strain from other Race 3 biovar 2 strains in Kenya. Ivey *et al.* (2007) concluded that rep-PCR method is very effective in distinguishing the *R. solanacearum* isolates below the sequevar level as compared to gene sequencing method. Norman *et al.* (2009) characterized *R. solanacearum* strains using rep-PCR element primers and reported its usefulness in distinguishing the clonal outbreaks of BW as it could be employed in classifying the isolates based on host, biovar and origin. Khakavar *et al*., (2008) clearly showed that *R solanacearum* strains were phylogentically similar within a region but diverse between regions despite biovar designation. Yu *et al*. (2003) distinguished the *R. solanacearum* strains isolated from ginger from local strains isolated from tomato (Race

1) and heliconia (Race 2) in Hawaii. Horita and Tsuchiya (2001) subjected 74 strains of *R. solanaceraum* from Japan to rep-PCR and differentiated biovar N2 strains which were grouped into 2 distinct groups, one belonging to Race 3 (Potato) and the latter to Race 1 (tomato, eggplant, pepper and tobacco isolates). *R. solanacearum* isolates from Taiwan, Japan and India did not show any correlation between the genetic variability, pathogenic virulence and the origin of the strain (Jaunet and Wang, 1999; Horita and Tsuchiya, 2001; Kumar *et al.*, 2004). Ramsubhag *et al.* (2012) separated the tomato isolates from central zone of Triniad compared to other regions employing the rep-PCR technique. It was observed that changes in the rep-PCR fingerprint patterns arise due to polymorphism, rearrangements, recombination or acquisition of foreign DNA (Ishii and Sadowsky, 2009) and is therefore regarded as an useful tool in the field of microbial biogeography (Cho and Tiedje, 2000).

1.9.4. Gene sequencing

The sequevar classification system is used in in identifying infrasubspecific groups of *R. solanacearum* (Fegan and Prior, 2005) and is perhaps the most powerful tool used by the researchers in assessing the genetic relationships among the *R. solanacearum* strains. Many authors have compiled the sequence data across the world (Ramesh *et al.*, 2014b; Castillo and Greenberg, 2007). In recent years, the 16S rDNA (Horita and Tsuchiya, 2000., Taghavi, *et al*, 1996), *egl* (Fegan, M and Prior, 2005), and *hrp*B (Poussier *et al.*, 2000) genes have played a crucial role in separation of *R. solanacearum* isolates into divisions that have helped in tracing the origin of these strains (Villa *et al*., 2005). Compared to other genomic regions (16SrRNA and tRNA), the *hrp* gene region, which is

involved in host-pathogen interactions, revealed the same major trend of diversity, suggesting that *hrp* genes have evolved in parallel with 16S rRNA and tRNA (Poussier *et al*., 1999).

1.9.5. MLST

In recent years, sophisticated approaches viz. multilocus sequence typing (MLST) sequencing (Castillo and Greenberg, 2007) or comparative genomic hybridization (CGH) (Guidot *et al*., 2007) is used in genetic diversity analysis of *R. solanacearum* isolates.

Wicker *et al*. (2012) used Multilocus sequence analysis (MLSA) to study the genetic diversity of *R. solanacearum* on a larger collection of strains. They observed many recombination events and ident6ified the populations that undergo diversification continuously. However, this analysis fails to differentiate appropriately between microvariations within the population (Maiden, 2006; Young, 2008). The hierarchical classification system of *R. solanacaerum* puts together all the strains belonging to Race 3 biovar 2 in a clade named sequevar 1 (Fegan and Prior, 2005).

Multilocus sequence analysis revealed that phylotype I is highly recombinogenic (Wicker *et al*., 2012). This high degree of diversity could be the due to its ability to naturally develop a state of competence resulting exchange of genetic material (Bertolla *et al*., 1999; Boucher *et al*., 1985). This gene acquisition and its role in defining the genetic diversity were clearly evident from the analysis of complete genome sequence of the *R. solanacearum* strain GMI1000 (Lerat *et al*., 2005). Diversity data of the clonal population proved that *R. solanacearum* created a wide range of variability in its genome.

Gophna *et al*. (2004) has reported *R. solanacearum* as the sole phytopathogen where horizontal gene transfer even from archaebacteria has been taken place.

1.9.6. PFGE and FAME

PFGE is generally accepted as one of the most powerful tools in diversity studies of *R. solanacearum*. Previous reports have indicated that fatty acid methyl ester (FAME) profiling corresponded well with DNA and rRNA homology and is known to be the best phenotypic marker for *R.solanacearum* strains classification (Dawyndt *et al*., 2006; Weller *et al*., 2000).

1.10. *R. solanacearum* and its genomic organization

The complete genome sequence of first *R. solanacearum* strain GMI1000 isolated by Bernard Digat from a wilted tomato plant in Guyana, and classified as Phylotype I, Race 1, biovar 3 has been determined and annotated (Salanoubat *et al.*, 2002) and found to have a bipartite genome structure (5.8Mb) with a high G+C content (average value of 67%) and a coding potential for approximately 5120 proteins (Genin and Boucher, 2004). Genome comprises of chromosome (3.7 Mb) and megaplasmid (2.1 Mb). Both replicons were described having mosaic structure since it contains regions in which the codon usage considerably differs from the average *R. solanacearum* codon usage. The genome sequence of twelve *R. solanacearum* strains is publicly available (Table 1.7). Very recently the genomes of two *R. solanacearum* strains, collected from India were also sequenced and annotated (Ramesh *et al.*, 2014a). The genome comparisons between all the available genome sequences confirmed that all the strains have a common ancestor.

Table 1.7 Details of *R. solanacearum* genomes (adapted from Remenant *et al.*, 2010; Genin and Denny, 2012; Ramesh *et al.*, 2014a)

No.	Strain	Origin	Host*	Phylotype	Sequence status	Reference
1	GMI1000	French Guyana	Tomato	I	Complete	Salanoubat *et al.*, 2002
2	Y45	China	Tobacco	I	Draft	Li *et al.*, 2011
3	K60	USA	Tomato	IIA	Draft	Remenant *et al.*, 2012
4	CFBP2957	French WestIndies	Tomato	IIA	Complete	Remenant *et al.*, 2010
5	IPO1609	Netherlands	Potato	IIB	Draft	Guidot *et al.*, 2009
6	UW551	Kenya	Geranium	IIB	Draft	Gabriel *et al.*, 2006
7	Molk2	Philippines	Banana	IIB	Draft	unpublished
8	Po82	Mexico	Potato	IIB	Complete	Xu *et al.*, 2011
9	CMR15	Cameroon	Tomato	III	Complete	Remenant *et al.*, 2010
10	PSI07	Indonesia	Tomato	IV	Complete	Remenant *et al.*, 2010
11	Rs-09-161	India	Eggplant	I	Draft	Ramesh *et al.*, 2014a
12	Rs-10-244	India	Chilli	I	Draft	Ramesh *et al.*, 2014a
13	P673	Taiwan	Ornamental plant	IIB	Complete	Bocsanczy *et al.*, 2014

1.11. Disease cycle of *R. solanacearum*

R. solanacearum naturally infects roots, penetrates through the cortexand latter disseminates throughout the vascular system (Vasse *et al.*, 1995). *R. solanacearum* has a strong tissue-specific tropism within the host as a result of which it rapidly multiplies in the xylem vessels soon after invading the host. Virulence factors idenified in the pathogenesis are the lytic enzymes (endoglucanases, pectic enzymes) EPS etc. (Meng, 2013). Inside the plant, the bacterium rapidly develops within intercellular spaces of the inner cortex; then, it crosses the natural barrier of the endodermis and penetrates into the vascular cylinder where it multiplies within vascular parenchyma to finally invade protoxylem vessels via cell wall degradation (Vasse *et al*., 1995). Extracellular polysaccharide production causes rapid wilting of infected plants as a result of accumulation of pathogenic bacteria (Saile *et al*., 1997; Timms-Wilson *et al*., 2001). The colonization of approximately 25% of xylem vessels in each vascular bundle above the collar zone is sufficient to induce partial wilting of tomato and ultimately leads to plant death (Sequeira, 1998). The functional analysis of pathogenicity genes indicates that several hydrolytic enzymes might be necessary to promote the intercellular progression of the bacterium within the inner cortex and during translation towards the xylem vessels (Denny, 2006; Genin, 2010). Under favorable conditions, symptoms include leaf flaccidity of the youngest leaves, yellowing of foliage, stunted growth of plant, browning of the xylem tissue takes place and the plant collapses with in 2-3 days.

In BW disease, milky-white exudation of bacterial cells from infected stem tissue is a prominent feature which is absent in fungal wilt diseases. . In case of tomato, sometimes infected plants does not show symptom until fruit ripening stage. Finally, it results in

rapid collapse of plant (Buddenhagen and Kelman, 1964). A longitudinal slice of infected stem and stolon revealed vascular browning with dark brown streaks. And in some cases grey-white bacterial oozing has been observed on stem surfaces (Buddenhagen and Kelman, 1964; Wallis and Truter, 1978). *R. solanacearum* may invade the susceptible host through microscopic wounds caused by the emergence of lateral roots (McCarter, 1991). Soon after the bacterial colonization, it produces extracellular polysaccharides which help in clogging the vascular tissue, resulting in death of the plant (Husain and Kelman, 1958). Under field conditions, the disease first appears in scattered patches on tomato. Wilting signs are first seen on younger leaves during hot weather. The vascular tissues of the stem show a brown discoloration and when stem is cut displays bacterial streaming that indicate the presence of dense masses of bacterial cells in infected vascular bundles (McCarter, 1991; Champoiseau *et al*., 2010). Browning of vascular system occurs in lower parts of the stem. In the tomato plants, the vessels remained occluded by intact tylosis for 48 to 72 h, such structural defense augmented by the partial blocking effect of tyloses after collapse. Sudden release of large number of bacteria from disrupted tylosis causes rapid and successful colonization in the xylem vessels and also found that the movement of the bacterium was more rapid in the vessels of the stem than in the root. In certain weed hosts viz. *Solanum dulcamara* infected with the pathogen might display discolouration of vascular tissue but no actual wilting (Champoiseau *et al*., 2010).

1.12. Pathogenicity testing

Vudhivanich (1997) has used micropipette technique for injection of various concentrations of *R. solanacearum* inoculums directly into the tomato plant by inserting

diagonally into the stem at the third leaf axil from the top. Seedling submergence technique for pathogenicity study of *R. solanacearum* was proposed by Marina and El-Nashaar (1993). In that, seedlings of tomato plant were treated with aqueous inoculums suspension of bacteria for 10 seconds and transplanted in field. The root severing and root drenching method were used to inoculate Capsicum plants with *R. solanacearum* (Wang and Berke, 1997). The injury was made to roots of 28 days old seedling and 30 ml bacterial suspension was inoculated into each pot (Wang and Berke, 1997). Inoculation of *R. solanacearum* on *Moringa oleifera* has performed by spraying the bacterial suspension onto pin pricked leaf axil of healthy plants and by dipping the cut ends of roots of the healthy plants in bacterial suspension (Estelitta *et al.*, 1997). It was observed that wilting symptoms in plants has been developed after 10 to 20 days of inoculation.

1.13. Virulence factors of *R. solanacearum*

Over the last three decades several important virulence factors were identified and characterized underlying *R. solanacearum* pathogenicity and virulence.

1.13.1. Exopolysaccharide

R. solanacearum produces a variety of extracellular products particularly one with high molecular mass acidic extracellular polysaccharide (EPS I) that contributes to disease symptoms. It is a heterogeneous polymer containing a trimeric repeat unit of N-acetylgalactosamine, 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 I is the most important virulence factor of *R.solanacearum*, since EPS mutants do not cause wilt

symptoms even when introduced directly into stem wounds although they remain slightly pathogenic (Denny and Baek, 1991).

EPS I-deficient mutants are known to poorly colonize the stem of infected plants which also reveals that EPS I plays a role to prevent or avoid the recognition of pili and/or lipopolysaccharide by plant defense mechanisms (Araud-Razou *et al.*, 1998; Saile *et al.*, 1997; Duvick and Sequeira, 1984).

1.13.2. Cell wall degrading enzymes (Cellulolytic and Pectinolytic enzymes)

R. solanacearum secretes three polygalacturonases (*PglA*, *PehB* and *PehC*) (Huang and Allen, 1997; Schell *et al.*, 1997) and an endoglucanase (*egl*) (Kang *et al.*, 1994), but gene disruption analysis proved that the role played by individual wall degrading enzymes in BW disease is negligible.

Egl mutants appear to be reduced in their ability to colonize the stems of infected plants but remain pathogenic. Another exoglucanase, a β-1, 4-exocellobiohydrolase, *CbhA*, that releases cellobiose from the non-reducing ends of the chains and it contribute almost as much to disease as *egl*, substantially in the ability of *R. solanacearum* to systemically colonize tomato plants (Liu *et al*., 2005; Roberts *et al.*, 1988).

R. solanacearum produces one pectin methylesterase (*Pme*), which helps in removal of methyl groups from pectin enabling the successive breakdown of cell wall by the three polygalacturonases (PGs). *R. solanacearum* has two types of PG: anendo-PG, named *PglA* or *PehA* (Schell *et al*., 1988; Allen *et al*., 1991), that cleaves the pectin polymer at random releasing large fragments, and two exo-PG, the exopoly-α-D-galacturonosidase

PehB (Allen *et al*., 1991) and exopolygalacturonase *PehC* (Gonzalez and Allen, 2003), that release galacturonic acid dimmers and monomers respectively.

1.13.3. Twitching motility (Type IV pili and fimbrial structures)

Liu *et al*. (2001) reported that *R. solanacearum* produces Type IV pili (Tfp) required for twitching motility that is composed mainly of a single pilin protein, PilA, assembled to a flexuous polar filament. Tfp is also responsible for its property of attachment to substrates and natural transformation (Kang *et al*., 2002). *R. solanacearum* type IV pili mutants were comparatively less virulent on host plants. A few factors viz. motility, adherence and/or type IV pili are known to contribute in *R. solanacearum* pathogenesis (Liu *et al*., 2001; Kang *et al*., 2002).Taken together, these results demonstrate the pilus formation promotes the attachment to host cell surfaces, colonises the root surfaces, migrates to wound sites (kang *et al.*, 2002).

Genin and Boucher (2004) reported the biofilm formation in plants by *R. solanacearum* and assumed that it helps bacterial survival during latent infections and saprophytic life.

1.13.4. Swimming motility

R. solanacearum can produce polar flagella (1-4) for swimming motility (Tans-Kersten *et al*., 2001). This ability is related with cell density and it was confirmed by Clough *et al*. (1997) who demonstrated that maximum number of bacteria exhibited motility in exponential phase as against the stationary phase that was comprised of non-motile bacteria. A soil soak pathogenicity assay on tomato plants with two non-motile mutants constructed by disrupting the *fliC* (encoding the subunit of the flagellar filament) and *fliM*

(encoding the flagellar motor switch protein) genes, showed a reduction in virulence of mutants compared to the wild-type strain, but this difference cannot be observed after wounded petiole inoculations, suggesting that swimming motility is the most crucial virulent factor that is essential during early stages of host plant invasion (Tans-Kersten *et al*., 2001).

1.13.5. Chemotaxis

Bacterial chemotaxis is the movement towards regions that contain higher concentrations of beneficial or lower concentrations of toxic chemicals and is required along with the motility for many pathogenic species to colonize and invade a host (Wadhams and Armitage, 2004). *R. solanacearum* uses its chemotaxis system to move towards more favourable conditions (Yao and Allen, 2006; Genin, 2010). Yao and Allen (2006) observed that *R. solanacearum* is more attracted by root exudates from the host plant tomato but it less attracted by rice exudates, hence they concluded that chemotaxis is an essential trait required for virulence in *R. solanacearum*. However, they observed that the non-tactic strains were as virulent as the wild-type strain, when inoculated directly into the stem, indicated that taxis is an important factor in the early stages for successful invasion of host tissues. The wild-type strain out-competed then on tactic mutants by 100 folds when co-inoculated (Yao and Allen, 2006).

1.13.6. The Type II Secretion System

Protein secretion plays an important role in virulence of many bacterial pathogens of plant and animals. *R. solanacearum* displays a remarkable ability for protein secretion

since more than 100 proteins can be identified in the cell-free supernatant of wild-type *R. solanacearum* cultured in minimal medium (Poueymiro and Genin, 2009). In *R. solanacearum* the plant cell wall degrading enzymes are secreted by the Type II secretion system, (also named as the General Secretory Pathway) a widely conserved Sec-dependent secretion pathway (Pugsley, 1993). The significance of the Type II secretion system was proved as the mutants defective in either system are severely impaired in colonization ability and multiplication *in planta* (Kang *et al*., 1994). After its entry in the plant, the bacterium must rapidly find nutrients to multiply and disseminate in the plant leaves.

1.13.7. The Type III Secretion System

Phytopathogenic bacteria employ type III secretion system (T3SS) inorder to suppress plant defense responses and this secretion system is encoded by the *hrp* (hypersensitive reaction and pathogenicity) gene cluster that translocates effector proteins into plant cells (He *et al*., 2004).

The HR basically prevents the the spread of pathogen infection by rapid death of cells thereby blocking it within the region adjacent to the surrounding infected area (Nimchuk *et al*., 2003). *R. solanacearum hrp* cluster encode the components of a type III protein secretion pathway (TTSP) (Van Gijsegem *et al.*, 1995), which plays a crucial role host pathogenesis (Boucher *et al.*, 1987). Population of *Hrp* mutant strains remains very low in the infected host plants than the wild type strains (Trigalet and Demery, 1986; Vasse *et al*., 2000) due to two factors i. e. low nutrient availability of nutrients and general plant defense responses (Genin and Boucher, 2004).

1.13.8. Lipopolysaccharide (LPS) and lectins

It is reported that the recognition between the pathogen *R. solanacearum* and the host involves an interaction between bacterial LPS and plant lectins (Whatley *et al*., 1980; Baker *et al*., 1984; Hendrick and Sequeira, 1984). *R. solanacearum* LPS is composed of lipid A, the oligosaccharide core (rhamnose, glucose, heptose, and 2-ketodeoxy-octonate) and the O-specific antigen (a chain of repeating rhamnose, N-acetylglucosamine, and xylose in a ratio of 4:1:1) (Baker *et al*., 1984). *R. solanacearum* strains that possess Smooth LPS (negative HR inducers) and rough LPS (positive HR inducers) basically indicated the presence or the absence of the O-specific antigen (Whatley *et al*., 1980; Baker *et al*., 1984). Kao and Sequeira (1991) have reported that LPS and EPS are co-related as the gene cluster was identified for the biosynthesis of cell surface components.

1.13.9. PhcA, a global regulator controlling phenotypic conversion (PC)

The production of virulence determinants in *R. solanacearum* are controlled by a regulatory network named PhcA (Brumbley *et al.*, 1993), which plays a role in activation of multiple virulence genes involving EPS biosynthesis, Pme and endoglucanase exoproteins, Type IV pili, and repression of genes involving production of polygalacturonases, siderophores, and motility (Schell, 2000; Kang *et al*., 2002; Genin and Boucher, 2002).

During early virulence stage, PhcA remains inactive at low *R. solanacearum* population resulting in inactivtation of polygalacturonases and both twitching and swimming motility. Whereas, during late virulence stage PhcA is actived at high *R. solanacearum* population that leads to production of EPS and essential cell wall degrading enzymes

(cellulases and pectin methylesterase) (Genin *et al*., 2005). This mechanism of PhcA is regulated by presence of a specific autoinducer molecule 3-hydroxypalmitic acid ester (3-OH PAME) (Flavier *et al*., 1997).

1.13.10. 3-OH PAME, an endogenous signal molecule essential to pathogenesis

3-OH PAME is synthesized by PhcB, a membrane-associated protein, from S adenosyl methionine. At high cell density in a restricted space, such as the plant vascular system, when extracellular 3-OH PAME accumulates above threshold concentrations (5 nM), it activates a two component regulatory system encoded by PhcS, a histidine kinase sensor, and PhcR, a response regulator (Clough *et al*., 1997). When inactive, this two-component system represses the production of PhcA (Flavier *et al*., 1997; Schell 2000). Therefore, when bacterial cells are in low density or are dispersed in the soil, levels of 3-OH PAME are low, consequently the two component system is inactive and PhcA levels are low. This results in the lack of expression of late virulence genes (EPS, cellulases) and the induction of expression of siderophore, pili and flagellar movement. On the other hand, when *R. solanacearum* cells are in high concentrations 3-OH PAME accumulation takes place which in turn triggers PhcS and PhcR, and accordingly elevates the PhcA levels in all cells. These bacterial cells become highly virulent due to abundant production of EPS I and exoenzymes (Genin *et al*., 2005; Schell, 2000).

1.13.11. Acyl homoserine lactone: a second Quorum sensing molecule

Acyl-homoserine lactones are autoinducers taking part in the quorum sensing (QS) system, a well-known mechanism of bacterial cell-cell communication that activates the

expression of the virulence genes only when bacteria are in high population levels (Fuqua *et al*., 2001). In the *R. solanacearum* regulatory network, PhcA positively controls the production of a second QS molecule, an Acyl homoserinelactone (acyl-HSL) dependent autoinduction system consisting of *luxR* and *luxI* homologues, designated *solR* and *solI* respectively (Huang *et al.*, 1995).

1.14. Factors influencing the survival of *R. solanacearum*

Survival and spread of the disease mainly depends upon the bacterial strain, its host, environmental conditions, agricultural practices and other factors that are not yet clearly identified (Boucher and Genin, 2004). Inspite of the fact that *R. solanacearum* is known to be soil borne in nature, its survival in soil remains very poor. However it is known to survive in soil (Buddenhagen, 1986); infected plant debris for prolonged periods (Grey and Steck, 2001); roots of asymptomatic weeds (Wang and Lin, 2005b); contaminated water (Caruso *et al.*, 2005; Danial *et al.*, 2006) and rhizospheres of non-host plants (Wenneker *et al*., 1999).

Several factors viz. pH, temperature, soil moisture content, salt level, soil type, soil microflora etc play a significant role in the *R. solanacearum* survival (Nesmith and Jenkins, 1985; Shekawat and Perombelon, 1991; Van Elsas *et al*., 2000; Van Elsas *et al*., 2001). All these factors interact amongst themselves affecting the overall *R. solanacearum* survival.

1.14.1. Temperature

Temperature requirements for disease development are different for different pathogens (Swanepol, 1990). The optimum temperature for growth of *R. solanacearum* is reported to be 28 °C. The disease severity is higher at 24-35°C; it occurs rarely in temperate climates. He *et al*. (1983) reported that a few strains grew at 37 °C. High temperatures (i.e. 30-35°C) promote occurrence of this disease, whereas soil temperatures below 20°C are not suitable for the disease (Gadewar *et al.*, 1999; Wang and Lin, 2005a). The maximum survival of *R. solanacearum* occurred at 12°C, 20°C and 28°C and was

independent on the cell density (Van Elsas *et al*., 2001). Stansbury *et al*. (2001) reported population decline of *R. solanacearum* Race 3 biovar 2 occurred at 15-20°C and at 4 °C. At a temperature of 32°C in controlled environment chambers, BW severity increased in two resistant tomato lines (Krausz and Thurston, 1975). Tung *et al*. (1990) reported that virulence factors are completely expressed at high temperatures. When soils were exposed to 43°C constantly for four days and over were free of the pathogen [International Potato Center (CIP), 1988]. Milling *et al*. (2009) suggested that the survival of *R. solanacearum* bv2 in temperate climates is related to its interaction with host plants rather than to its persistence at low temperature in the open environment. Temperature regime might also affect both growth and virulence of strains. Pathogen survival is reduced by temperature extremes, pH extremes and the presence of salts or other contaminants (Caruso *et al.*, 2005; Van Elsas *et al*., 2001). Low temperature is an important factor that triggers the conversion of *R. solanacearum* cells to the VBNC form. Recently, *R. solanacearum* biovar 2 was experimentally shown to become unculturable in two different soils during incubation at 4°C (Van Elsas *et al*., 2000). Prolonged but constant low-temperature stress appeared to broadly weaken *R. solanacearum* (Scherf *et al*., 2010). Hot and humid conditions during monsoon season (July to August) are most favorable for development of this disease in Pakistan (Burney, 1995). Studies conducted in Taiwan and India showed that the frequency of BW was 50-100 % during summer months (Wang and Lin, 2005a). Graham and Lloyd (1979) reported that survival of *R. solanacearum* (Race 3) was maximum in deeper layers of soil.

The most favourable conditions for optimum growth of *R. solanacearum* are high temperatures and high soil moistures, but certain strains that belong to Race 3 also thrive

well, at low temperatures (Stevenson *et al*., 2001). Granada and Sequeira (1983) has proved that *R. solanacearum* can even survive at 4°C in plastic bags for a period of 673 days.

1.14.2. Soil moisture

Soil moisture is significant factor that is essential for the pathogen multiplication. Nesmith and Jenkins (1985) reported that most favorable soil moisture is -0.5 to -1 bar while -5 to -15 bars are unfavorable. High soil moisture accumulates as a result of high water table or heavy rainfall which usually favors disease (Kelman, 1953). Survival of the pathogen is maximum in wet and well-drained soils, as against soil aridness and water logging (Buddenhagen, and Kelman, 1964; Kelman, 1953).

R. solanacearum population gets reduced in uncultivated land but certain soils donot have this effect.

1.14.3. Soil Type

Soil types play a critical role, as they influence the soil moisture. Soil type influences the structure of microbial communities, and determines the habitable niches in soil (Garbeva *et al*., 2004) and provides masses of diverse microhabitats.

It is generally accepted that clay soils favor bacterial activity (Alabouvette *et al.*, 1996) explaining that clay or heavy soils are conducive to BW (Hayward 1991). Power (1983) observed that *R. solanacearum* did not survive in alkaline soils. Yamazaki and Hoshina (1995) reported that Calcium nutrition affects the expression of BW in tomato as in when the calcium levels are increased BW disease symptoms are suppressed. Similarly, the

sticky and wet soils are known to be highly suppressive to *R. solanacearum* (Parish and Feillafe, 1965). These conditions are suitable for the growth of antagonistic microorganisms.

Soil texture also influences soil structure, through the distribution of different pore sizes, determining the actual living space for bacteria, fungi and predators, which further influences the survival of *R. solanacearum*. It also influences the water activity; water retained in pores of narrow diameter being less available for organisms that water present in big pores.

Nesmith and Jenkins (1985) observed that some soils in wilt-endemic areas have never permitted the survival of *R. solanacearum* even on artificial inoculation in the soil (Kelman, 1953; McCarter, 1976)

Elsas *et al*. (2000) studied the survival of *R. solanaceraum* biovar 2 isolate in loamy sand and 2 different silt loam soils and observed that *R. solanacearum* population declined at 20° C in loamy sand soil. Abdullah *et al.* (1983) reported severe infection in heavy clay soil. Similarly, Kelman (1953) too reported the BW severity on peanuts in heavy clay soils in Indonesia. Moffet *et al*. (1983) explained that *R. solanacearum* gets adsorbed on clay particles and survives from microbiostasis and therefore the pathogen population gradually falls down. Keshwal *et al.* (2000) reported that minimum population of *R. solanaceraum* PST4 persisted in sandy loam soil due to the presence of high content of sand and low content of silt that caused 32.2% tomato wilt.

1.14.4. Salinity

Salts play an important role in the life of organisms. Its concentration in seawater is 3-5% (or 0.5-0.85M). The requirement of salt varies from organism to organism. Van Elsas *et al*. (2001) reported that the levels of seawater salts in coastal areas proved to be harmful to the survival of the pathogen. This was again proved by incubating *R. solanacearum* biovar 2 strain 1609 in water containing 100 mM NaCl, 10 mM $CaCl_2$ and 3.3 mM $MgSO_4$ salts (Van Elsas *et al*., 2001). The concentrations of these salts were representative for sea-water and indicated that *R. solanacearum* cells are stressed by exposure to low water activity.

1.14.5. Light Intensity

Van Elsas *et al*. (2001) reported that both incident light and seawater salts were detrimental to pathogen survival. Krausz and Thurston (1975) also concluded that there was a reduction in the resistance of line 1169 to isolate LB-6 with the reduction of light intensity irrespective of the temperature. Sequeira and Rowe (1969) showed that higher the light intensity (2000 ft-c.) higher was the resistance expressed in potato.

1.14.6. Nematode Populations

The positive interaction between *Meloidogyne* spp. incidence and *R. solanacearum* has been studied (Kelman, 1953; Martin and Nydegger, 1982; Reddy *et al*., 1979). Deberdt *et al*. (1999) reported that there is a high degree of correlation between root knot nematode and the degree of BW infection. High root galling index reflects the higher percentage of wounding of root and thereby providing easy access to *R. solanacearum* for colonization. (Reddy *et al.*, 1979).

1.14.7. Native Soil microbiota

Soils host complex biological communities where microorganisms prevail in number and diversity. Some prokaryotes use the rhizosphere and/or the rhizoplane as their ecological niches, where they survive, multiply, and find protection against the antagonistic surrounding microflora. Such microorganisms have been generically called rhizobacteria (Beauchamp, 1993). Rhizobacteria are potential agents for the biological control of plant pathogens (Ramesh *et al.*, 2009; Wei *et al.*, 1996; Zehnder *et al.*, 2001).

1.14.8. Nutrient content of the soil

Nutrient concentration in soil was found affecting bacterial growth and disease development in plant (Lucas, 1975; Kang *et al.*, 2004). High concentration of nitrate depresses the growth of pathogen and wilt development in tobacco (Lucas, 1975). Similarly, increased phosphate content of soil was found negatively correlates to the disease incidence (Kang *et al.*, 2004).

1.15. Transmission and survival of *R. solanacearum*

Dispersal of *R. solanacearum* on vegetatively propagation material has been known for many years. *R. solanacearum* travels longer distance via vegetative propagating material. Pathogen enters into other countries by the introduction of *R. solanacearum* Race 3 (R3) into Europe and North America by geranium cuttings imported from Kenya (Swanson *et al.*, 2005). Seed transmission has been demonstrated in groundnut and artificially contaminated seeds of tomato and capsicum were also reported to transmit the disease (Moffett *et al.*, 1981). Kumar *et al.* (2004) indicated that *R. solanacaerum* biovar 3 strains belonged to single ancestry and probably spread through rhizome. The excessive

use of infected ginger rhizomes within China, Indonesia and Malaysia has led to major dissemination of BW (Lum, 1973).

BW of potato has been spread locally and internationally on latently infected potato tubers (Ciampi and Sequeira, 1980; Ciampi *et al*., 1980; Sunaina *et al*., 1989). Latent infection has emerged in the past two decades as a most insidious aspect of disease biology of great concern to plant breeders. In potato, pathogen disseminates through infected seed tubers, weeds, irrigation water, contaminated tools and infested soils (Hay, 2001) and sometimes through insects (Stevenson *et al*., 2001).

R. solanacearum is usually disseminated by soil, surface water, cultural practices, infected transplants, tubers, tools, insects, nematodes and humans. Insects are very important for epidemic development, mainly honey bees and wasps that transport infected sap from male inflorescences and bract scars to newly formed scars which are susceptible until 2 days after tissue exposure. The survival of *R. solanacearum* in soil and water is due to its association with either reservoir plants or plant debris (Hayward, 1991; Wenneker *et al*., 1999; Van Elsas *et al*., 2000, Norman *et al*., 2009).

Survival and dissemination of *R. solanacearum* is generally enhanced by high soil water content. Olsson (1976) reported that use of contaminated surface water for irrigation could be a source for dissemination of *R. solanacearum*. Wang and Lin (2005) has showed that the disease often spreads through irrigation water, infested soil, farm implements or even shoes of farm personnel and its prevalent in low lying areas with prolonged high soil moisture. Irrigation water contaminated with *R. solanacearum* has been responsible for outbreaks of BW on a number of crops. In the case of potato, water effluents resulting from domestic or industrial processing of this material led to

contamination of water streams (Elphinstone *et al.*, 1996; Janse, 1996). For example seawater salts inhibited the survival of *R. solanacearum* due to osmotic tensions (Van Elsas *et al*., 2001; 2005). In surface water layers, incident light in a light-dark regime had a detrimental effect on *R. solanacearum* survival, presumably due to either a direct effect on cells caused by photo-oxidative damage or an indirect effect by stimulating growth of algae which might be competitors or antagonists of the pathogen (Van Elsas *et al*., 2001; 2005).

R. solanacearum can survive in the soil; however it is evident that this pathogen does not survive in bulk soil away from plant material except for short periods of time (Graham and Lloyd, 1979). Van Elsas *et al.* (2000) reported a decrease in bacterial population in the three different fields evaluated over the time. The survival time of this pathogen in this habitat has been a point of differentiation between different researches (Coutinho, 2005). Other evidences support that long-term survival of *R. solanacearum* in the soil could be due to the fact that the pathogen inhabits and colonises the weeds and non-hosts (Graham and Lloyd, 1979; Granada and Sequeira, 1983) as studied by Quimio and Chan, (1979) and Granada and Sequeira (1983). The fact that several weeds growing either during the winter or summer seasons can serve as carriers of the pathogen (Pradhanang *et al*., 2000) revealed the possibility for persistence of the pathogen in the field during intercropping periods. A few of them have been listed in the following Table 1.8.

Table 1.8 List of Weeds and symptomless hosts

No.	**Weeds and Symptomless hosts**	**Reference**
1	*Croton hirtus*	Abdullah, 1982;
2	*Solanum carolinense*	Dukes *et al*., 1965

No.	Weeds and Symptomless hosts	Reference
3	*Lagasca mollis*	Kishun *et al*., 1980
4	*Portula caoleracea*	Moffett and Hayward, 1980; Quimio and Chan, 1979
5	*Solanum dulcamara*	Elphinstone *et al.*, 1997
6	*Drymaria cordata* and *Polygonum capitata*	Pradhanang *et al*., 2000
7	*Ageratum conyzoides* and *Ranunculus scleratus*	Sunaina *et al*., 1989
8	*Solanum cinereum*	Graham and Lloyd, 1978
9	*Solanum dulcamara*	Sequeira and Kelman,1976
10	*Urticadioica* and *Solanum dulcamara*, *Tagetes minuta*	Wenneker *et al.*, in 1999
11	*Alternanthera sessilis* and *Physalis minima*	Ramesh *et al.*, 2011a

According to Coutinho (2005), factors like soil moisture content, temperature and soil type can all play a critical role in the survival of *R. solanacearum* and also depends on its own physiology and physical requirements as well as the interactions between these factors. Survival is dependent on the race; Race1 is persistent for more number of years due to larger number of hosts, while Race 3 biovar 2A survives for a limited period due to restricted number of hosts (Martin and French, 1985; Champoiseau *et al*., 2009). In addition, it has been proposed that the viable but non culturable state (VBNC) of *R. solanacearum* may be also involved in longer persistence of pathogen in water and soil and at different stages in plant infection (Oliver, 2005; Grey and Steck, 2001).

1.16. Detection of *R. solanacearum*

The rapid and sensitive detection of the pathogen is very important to curb the disease. Bacterial ooze is noticed when cut end of stem from an infected plant is placed into a glass of water (Smith *et al*., 1997). This is the primary diagnostic tool for determine the BW. Following are the methods that used for the identification of the pathogen (Table 1.9) in brief.

Table 1.9 Various methods for detection of *R. solanacearum* isolates

No.	**Method**	**Techniques**	**References**
1.	Traditional methods	Bacterial streaming test	Allen *et al*., 2001
		Semi-selective medium (SMSA)	Denny *et al*., 2001; Elphinstone *et al*., 1996
		Non-selective medium	Caruso *et al*., 2002
		Culture from Tissue extracts	Elphinstone *et al*., 1996; Pradhanang *et al*., 2000b
		From soil (VBNC state)	Denny *et al*., 2001; Pradhanang *et al*., 2000b
2.	Serological methods	ELISA tests	Pradhanang *et al*., 2000b
		ELISA on enriched sample	Elphinstone *et al*., 1996; Pradhanang *et al*., 2000b
		Double antibody Sandwich ELISA technique (DAS–ELISA)	Priou *et al*., 2006
		Immunofluorescence-Antibody staining (IF-AS) and Immunofluorescence-colony staining (IF-CS)	Elphinstone *et al*., 1996; Janse, 1988
		Monoclonal antibody (8B-IVIA)	Caruso *et al*., 2002

		Techniques	**References**
		immunostrips	Swanson *et al.*, 2007
		Serological kits: e.g., Agdia, Neogen	Agdia, Inc., Elkhart, Indiana, USA
3.	Nucleic Acid based methods	Direct PCR	Ramesh *et al*., 2011a; Khakvar *et al.* 2008; Elphinstone *et al*., 1996; Weller *et al*., 2000; Poussier *et al.*, 2002; Ozakman and Schaad, 2003
		Nested PCR	Elphinstone *et al*., 1996 Pradhanang *et al*., 2000b
		Co-operational PCR	Caruso *et al*., 2003
		Real-time PCR	Kutin *et al.*, 2009; Ozakman and Schaad, 2003; Weller *et al*., 2000;
		Loop mediated amplification (LAMP)	Kubota *et al.*, 2008
		BIO-PCR	Ramesh *et al.*, 2011b

1.17. Management of BW

It is very difficult to control BW caused by *R. solanacearum* in field production on crops, since the pathogen is soil borne bacterium and has a broad host range in addition to the genetic variation level within the strains (Saddler, 2005; Denny, 2006). A number of methods are devised for the same by the researchers such as Resistance breeding, chemical control and biological control. All these methods are known to control the disease but not a single method provides complete control of BW (Melton *et al.*, 2004). Therefore, for effective management of disease all different strategies like crop rotation, nematode management, chemical application, resistant cultivar and proper cultivation, stalk and root destruction after harvest should be used efficiently (Lucas, 1975; Shew and Lucas, 1991; Fortnum and Martin, 1998).

Effective and long term disease managment is possible in integrated approach including the use of resistant/tolerance varieties, chemical and biological control and cultural practices as parts of an integrated pest management strategy.

1.17.1. Breeding for disease resistance

Genetic enhancement of crop plants for improved resistance is priority of the researchers working on this disease. Variability of pathogen genotypes and lack of information on BW resistance sources are among major challenges faced by breeders. Worldwide many international organizations i.e. Asian vegetable Research and Development Centre, Taiwan (AVRDC, also called world vegetable centre, focusing on solanaceous crops, International Potato Center (CIP focusing on potato) and oil crops Research Institute,

China (OCRI focusing on peanut) are working in close collaboration with many developing and developed countries around the world.

Host Resistance is strongly influenced by environmental conditions (soil temperature, pH, and moisture) (Scott *et al*., 2005). Moderate level of host resistance to BW is achieved for most solanaceous crops especially when conditions are not extreme (Champoiseau *et al*., 2010). In the past few years, much research work has been done to develop resistant varieties (Table 1.10).

Table 1.10 List of resistant varieties of eggplant, tomato, potato and pepper.

No.	**Crop**	**Varieties**	**Reference**
1	Eggplant	SM 6-6, Arya Nidhi, Arka keshav, Arka neelkanth	Chaudhary and Sharma, 1999;
		Swetha, Surya SM 141,	Gopalakrishnan *et al.*, 2005
		DWD x Malapur and DWD x Rabakavi	Ajjappalavara *et al.*, 2008
2	Tomato	Hawaii 7996	Wang *et al.*, 2000
		CRA 66	Grimault and Prior, 1993
3	Potato	*S. phurejia* and *S.cummersonii* hybrids	Laferriere *et al.*, 1999
		S. phureja resistant lines	French and Lindo, 1982; Sequeira, 1979
4	Pepper	IHR-546 and PBC 631	Singh and Sood, 2004

1.17.1.1. Eggplant

First reports for resistance sources in eggplant came from Puerto Rico. Ali *et al. (*1990) reported the presence of certain wild relatives of eggplant which are resistant or totally immune to BW. Collonnier *et al*. (2001) produced somatic hybrids of *S. melongena* and *S. aenthiopicum* using protoplast electrofusion. It was found that all somatic hybrids produced in this way were tolerant to BW.

1.17.1.2. Pepper

Many resistance sources have been identified in various types of pepper worldwide (Hartman and Elphinstone, 1994; Matsunaga and Monma, 1999). Quezado Soares and Lopez (1995) reported that capsicum germplasm originating from Asian countries were more resistant to BW as compared to those from European or American countries.

1.17.1.3. Potato

Potato Research Centre (CIP) has initiated several breeding programs with special emphasis to resistance against latent infection. Hybrids between *Solanum commersonii* and *S. tuberosum* is shown to be resistant to *R. solanacearum* and are being used for further breeding efforts (Carputo *et al*., 2009).

1.17.1.4. Tomato

Resistant tomato cultivars (e.g. FL7514 and BHN 466) are known to provide moderate resistance against BW and are now commercially available (Champoiseau *et al*., 2010). Grafting susceptible tomato cultivars onto resistant root stocks is a successful method in tomato wilt management in various parts of the world (Saddler, 2005).

1.17.1.5. Tobacco

In tobacco, Robertson *et al*. (2004) has reported a resistance gene to develop BW resistance.

1.17.2. Grafting

Grafting is identified as a useful tool in the management for BW. A resistant variety of tomato CRA 66 has been recognized in India and Germany (Grimault and Prior, 1993).

1.17.3. Cultural methods

Researchers have been successful in managing BW by making use of crop rotation practice. This approach of crop rotation involves the rotation of crops into an agricultural system that eventually leads to decline in pathogen number in the soil. In Nepal, crop rotation of a susceptible tomato cultivar was practiced with corn, lady's finger, cowpea and a partly resistant tomato cultivar (Adhikari and Basnyat 1998). This practice resulted on reduction in BW severity and delayed the infection by 1-3 weeks for cultivars. Melton and Powell (1991) also reported that cohesive practice involving resistance and crop rotation could bring better results in disease management. Crop rotation for two years could reduce the disease incidence in heavily infested potato fields, and was also effective for a period of a one-year (Lemaga *et al*., 2001).

Intercropping of different time length with several plant species such as bean, cabbage, cowpea, onion, pea, or several cereals like maize or wheat has proved variable efficiency in BW control (Devaux *et al*., 1987; French, 1994; Dhital *et al*., 1996; Terblanche, 2002; Katafiire *et al*., 2005; Lemaga *et al*., 2005). Other methods include, planting healthy

(tested) seed, using of cover crops and other measures to reduce the impact of weed hosts as herbicides, since there are many considered as asymptomatic to *R. solanacearum* infection (Lopez and Biosca, 2005), and avoidance or testing and treatment of surface water for irrigation (Champoiseau *et al*., 2010).

For managing BW good sanitation methods and monitoring of weeds is very crucial. Crop rotation may be able to reduce bacterial populations in the soil too. Saddler (2005) reported that cultural control methods work at it's the best in regions where the disease is endemic. Champoiseau *et al*. (2010) also agrees that in the regions where BW of potato is endemic cultural methods can be effective under some conditions as they can reduce disease, including crop rotation which dependent on factors such as the ability of the local *R. solanacearum* strains to survive and maintain a remaining infective population in the absence of a host (Mariano *et al*., 1998).

1.17.4. Chemical control

Direct control of BW diseases caused by *R. solanacearum* in the field has showed to be difficult due to the fact that the bacterium localizes inside the plant xylem and is able to survive at depth in soil (Mariano *et al*., 1998). One of the commercially-available chemical formulations is acibenzolar-S-methyl, which induces the systemic acquired resistance (SAR) and activates the defense pathway.

Further the disease control can be carried out by treating the contaminated water with chlorine or peracetic acid. Disinfestation of the soil can be done with vapam, methyl bromide, or chloropicrin. Ji *et al*. (2005) has reported the use of thymol, palmrosa oil, products of plant origin can be used as biofumigants in BW control on tomato. A soil

drench consisting of phosphorous acid led to elimination of BW incidence in ornamental geranium (Norman *et al*., 2006) by acting as a bacteriostatic compound in the soil, as it was found to inhibit growth of *R. solanacearum in vitro* (Norman *et al*., 2006).

1.17.5. Biological control

Biological control measures are becoming common in integrated disease management (IDM). Research has been developed in trying to come up with strategies involving use of bacteria and plants to tackle the BW. Plant growth promoting rhizobacteria (PGPR), especially endophytes from eggplant, cucumber and groundnut are having been investigated for their role in the control of BW disease (Ramesh *et al.*, 2009). Most of the selected antagonists produced an antibiotic, DAPG, which inhibited *R. solanacearum* under *in vitro* conditions and *in vivo* conditions. Similarly, in case of tomato plant *P. fluorescens* was found effective for control of BW disease (Vanitha, *et al.*, 2009). Fluorescent pseudomonads exhibited biocontrol activity against *R. solanacearum* (Megha *et al.*, 2007). Furthermore, talc based formulations are prepared and added in the root zone (Guo *et al*., 2004). Incorporation of arbuscular mycorrhizal fungus (*Glomus versiforme)*, reduced the *R. solanacearum* populations in the rhizosphere, root surfaces and in the xylem (Zhu *et al.*, 2004). *Pythium oligandrum* (PO) is an effective biological control agent and acts as a mycoparasite on a diverse range of fungi due to the elicitor activity of the cell wall proteins (CWP) and on application to tomato roots enhanced the resistance of the host against BW. Research have been developed in trying to come up with plants that can be grown with potato to reduce BW incidences, coming up with positive beneficial microbes to counter soil pathogens, and incorporation of some parts of

the plant to inhibit survival of pathogens. BW can be controlled by incorporation of about 20% of fresh aerial parts of *Cajanus cajan* (Cardoso *et al*., 2006) and *Geranium carolinianum* (Ooshiro *et al.*, 2004). Messiha (2006) found out that *Stenotrophomonas maltophilia* had more inhibiting effects on *R. solanaceraum* in Egyptian clay soils than in Dutch soils.

1.17.6. Botanicals in the suppression of BW

Currently, researchers have diverted a lot of attention towards the plants as they are known to produce a diverse range of bioactive molecules. Thus it is a logical approach in drug discovery identifies the plants having antibacterial properties. Plant extracts and secondary metabolites from plants have been used by many researchers in disease control over synthetic bactericides (Abera *et al.*, 2011). Scientists have tapped on the use of plant by-products that imbibe the antimicrobial properties, on several pathogenic bacteria and fungi (Bylka *et al.*, 2004; Shimpi and Bendre, 2005; Kilani, 2006). Hassan *et al.* (2009) reported the potential of aqueous extracts of *Hibiscus sabdariffa*, *Punica granatum*, and *Eucalyptus globulus* against bacterial wilt under greenhouse and field conditions in potato plants. Deberdt *et al.* (2012) have reported the *in vitro* inhibition of *R. solanacearum* phylotype IIB/4NPB by *Allium fistulosum* extracts. Abo-Elyousr and Asran (2009) reported that *A. sativum*, *Datura stramonium* L. and *Nerium oleander* L. extract successfully reduced tomato bacterial wilt incidence on soil application *in vitro* tests

Wagura *et al.* (2011) proved the efficiency of crude medicinal plant extract of *Ocimum gratisimum*, *Brassica oleracae* and *Ipomoea batatas* on *R. solanacearum*. Extracts of

Myrtus communis, *Lantana camara*, *Cassia sp.* (Moussa *et al.*, 2010) and *Morinda citrifolia* (Sunder *et al.*, 2011) were found to be very effective against the pathogen. Inhibition of *R. solanacearum* by plant extracts have been reported recently (Lemos *et al.*, 2005; Lopez *et al.*, 2005; Larkin and Griffins, 2007; Walters, 2009). Sangoyomi *et al.* (2011) reported that aqueous extracts of medicinal plants failed to inhibit the pathogen growth and therefore it would be wise to study the effects of different solvent extracts other than water in repressing the growth of *R. solanacearum.* Methanol extract of sawdust of sugi *(Cryptomeria japonica)* heartwood inhibited the growth of *R. solanacearum* (Matsushita *et al.*, 2006).

Plant extracts are found to be eco-friendly alternatives and, thus, display a potential source of integrated pest and disease management programs.

Chapter II

Diversity of *Ralstonia solanacearum* infecting eggplant from Goa and adjoining regions and its characterization

2.1. Introduction

BW caused by *R. solanacearum* (Smith, 1995) Yabuuchi infects numerous plant species and is widely distributed in different regions of the world (Xu *et al.*, 2010; Champoiseau *et al.*, 2009). *R. solanacearum* cells proliferating rapidly at the sites of infection, invade the intercellular spaces of the root cortex, followed by colonization of vascular parenchyma eventually leads to necrosis and vascular browning resulting in death of the host (Vasse *et al.*, 1995; Denny *et al.*, 1990).

Members of *R. solanacearum* comprise a comparatively assorted group of isolates referred to as a species complex (Gillings and Fahy, 1994). *R. solanacearum* is classified into 5 races (Buddenhagen *et al.*, 1962) based on the host range; 6 biovars (Xue *et al.*, 2011) based on the metabolic profiles related to the ability to reduce carbohydrates and 4 phylotypes (I, II, III and IV) based on the ITS region, *hrpB* gene and *egl* gene sequences (Fegan and Prior, 2005).

Many studies have been done out to study the phylogeny of *R. solanacearum* using PCR-RFLP (Villa *et al.*, 2003), AFLP (Poussier *et al.*, 2001) and rep-PCR (Ivey *et al.*, 2007) to determine group clustering in *R. solanacearum.* Researchers are making use of techniques such as multilocus sequence analysis (MLSA) and comparative genomic hybridization (CGH) (Guidot *et al.*, 2007) to study the genetic diversity of *R. solanacearum* isolates (Castillo and Greenberg, 2007; Liu *et al.*, 2009). The pathogen is regarded as a clonal organism following estimations of linkage disequilibrium between

loci (Castillo and Greenberg, 2007). Many authors have also suggested the role of recombination and horizontal gene transfer in the evolution of this pathogen (Coupat-Goutaland *et al.*, 2011). In the recent years, publicly available full genome sequences of 13 strains of *R. solanacearum* have opened up several genomic choices that can be exploited for diversity analysis of this complex bacterial species (Ramesh *et al.*, 2014a; Remenant *et al*., 2012; Xu *et al.*, 2011, Li *et al.*, 2011; Salanoubat *et al.*, 2002).

R. solanacearum mainly affects the solanaceous vegetables in India (Singh *et al.*, 1997) and is prevalent in Meghalaya, Tripura, Mizoram, Gujarat, Western Maharashtra, Karnataka, Kerala, Madhya Pradesh, Assam, Orissa, West Bengal, , Manipur, Arunachal Pradesh, Andaman and Nicobar Islands (Ramesh *et al.*, 2014a; Shekhawat *et al.*, 1978). Eggplant cultivation in Goa is severely affected due to bacterial wilt and the incidence ranges from 30-100% (Ramesh, 2006).

In order to devise successful breeding and integrated management programme a detailed study of the pathogen diversity is a must (Sanchez Perez *et al.*, 2008). The objective of this study was to isolate the pathogen from different geographical locations of Goa and Maharashtra, phenotypically characterize them and determine the genetic diversity by various methods.

2.2. Materials and methods

2.2.1. Isolation of *R. solanacearum* and it maintenance

2.2.1.1. Collection of wilted samples

BW disease is known to occur during the cropping season from September to March. Wilted samples (Fig. 2 A) of diseased crops (Eggplant, tomato and Chilli) were collected

from Goa state which is located on the west coast of India and lies between 14°53′54″ and 15°40′00″ N latitude and between 73°40′33″ E and 74°20′13″ E longitude.

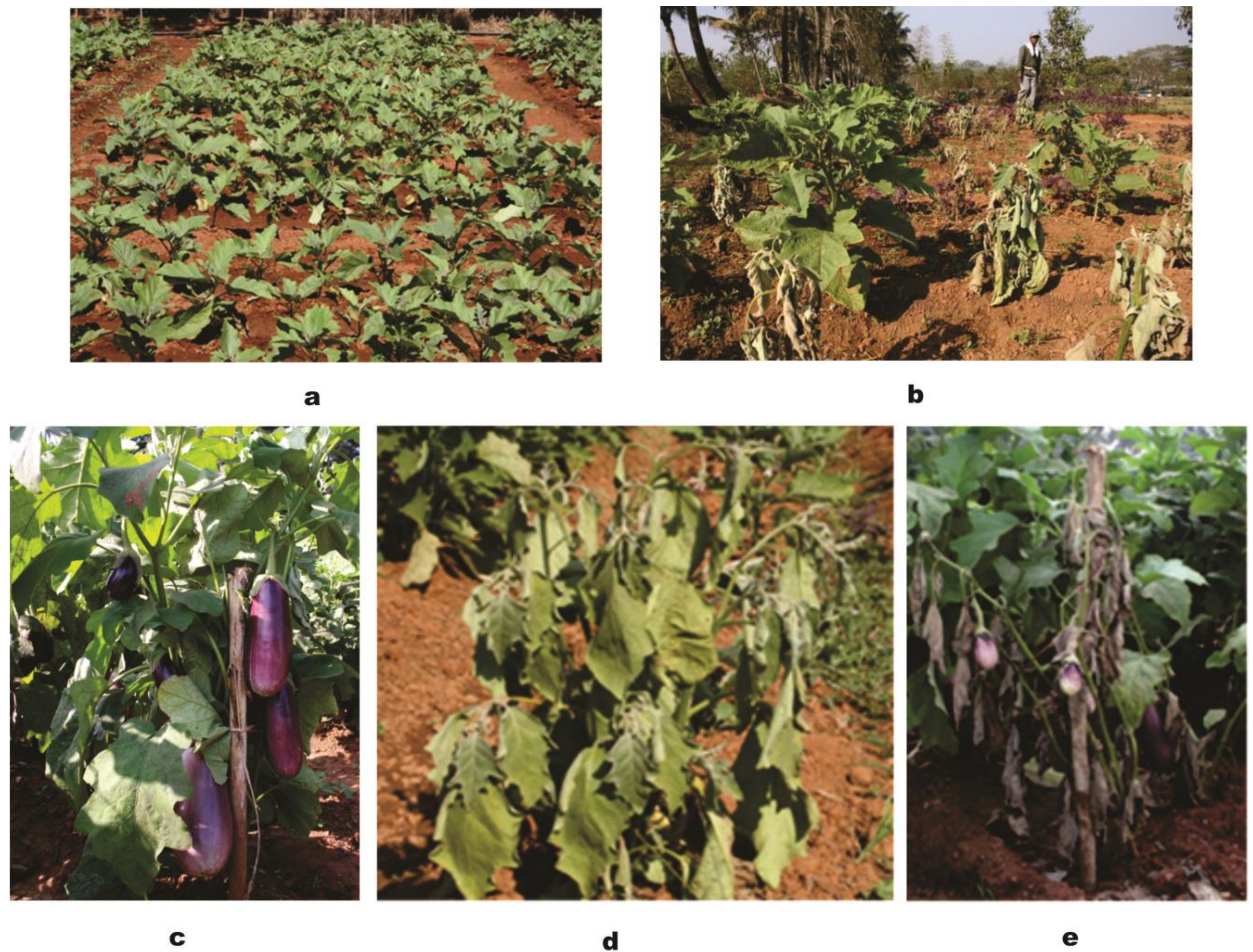


Fig. 2 A. BW disease symptoms: (a) Disease free field; (b) field severely infected with disease; (c) healthy eggplant; (d) early stage of the disease showing leaf drooping symptoms; (e) completely wilted and dead eggplant.

In the present study, 9 Talukas of Goa were identified for collection of wilted plants in the vegetable growing areas during the months of December and January for 4 consecutive years from 2008-2011. Plant samples were also collected from Maharashtra and a few isolates were procured from Culture collection of Plant Pathology Laboratory, ICAR Research Complex for, Goa from different states for comparative study. Plant

samples were placed in polyethylene bags, labeled and transferred to the laboratory. A total of 50 isolates were used in this study (Table 2 A)

Table 2 A. List of *R. solanacearum* isolates used in this study

No.	Isolate	State	Taluka	Place of isolation[a]	Host	Year[#]
1	Rs-08-01	Goa	Tiswadi	Pilar	Eggplant	2008
2	Rs-08-17	Goa	Tiswadi	Sulabhat	Eggplant	2008
3	Rs-08-43	Goa	Tiswadi	Caranzalem	Eggplant	2008
4	Rs-08-44	Goa	Tiswadi	Mansher	Eggplant	2008
5	Rs-08-47	Goa	Tiswadi	Mansher	Eggplant	2008
6	Rs-08-49	Goa	Tiswadi	Caranzalem	Eggplant	2008
7	Rs-08-61	Goa	Tiswadi	Caranzalem	Eggplant	2008
8	Rs-08-74	Goa	Tiswadi	Taligoa	Eggplant	2008
9	Rs-09-83	Goa	Pernem	Kasar	Tomato	2009
10	Rs-09-84	Goa	Tiswadi	Caranzalem	Eggplant	2009
11	Rs-09-86	Goa	Tiswadi	Caranzalem	Eggplant	2009
12	Rs-09-104	Goa	Tiswadi	Taligoa	Eggplant	2009
13	Rs-09-113*	Kerala	Trichur	Vettingapadam	Chilli	2009
14	Rs-09-123*	Kerala	Kottapalum	Kottapalum	Eggplant	2009
15	Rs-09-153	Goa	Canacona	Upta	Eggplant	2009
16	Rs-09-157	Goa	Mormugoa	Mestawada	Eggplant	2009
17	Rs-09-172	Goa	Tiswadi	Malawara	Eggplant	2009
18	Rs-09-173	Goa	Tiswadi	Malawara	Eggplant	2009
19	Rs-09-176	Goa	Tiswadi	Malawara	Eggplant	2009
20	Rs-09-189*	Karnataka	Hassan	Salagame Hobli	Potato	2009
21	Rs-09-197*	Karnataka	Mysore	Udduru	Chilli	2009
22	Rs-09-201*	Karnataka	Chikballapur	Nagalmuddama	Chilli	2009
23	Rs-10-209*	Kerala	Thrissur	Pachikere	Tomato	2010
24	Rs-10-216*	Kerala	NA	Calicut	Ginger	2010
25	Rs-10-250*	Andaman	NA	Andaman	Eggplant	2010
26	Rs-10-262	Goa	Tiswadi	Mansher	Eggplant	2010
27	Rs-10-266	Goa	Tiswadi	Nagali	Eggplant	2010
28	Rs-10-268	Goa	Tiswadi	Saplaan	Eggplant	2010
29	Rs-10-273	Goa	Tiswadi	Taligoa	Eggplant	2010

No.	Isolate	State	Taluka	Place of isolation[a]	Host	Year[#]
30	Rs-10-277	Goa	Tiswadi	Mandur	Eggplant	2010
31	Rs-10-278	Goa	Tiswadi	Pilar	Eggplant	2010
32	Rs-10-280	Goa	Ponda	Keri	Chilli	2010
33	Rs-10-286	Goa	Bicholim	Amona	Eggplant	2010
34	Rs-10-295	Goa	Bardez	Guirim	Eggplant	2010
35	Rs-10-298	Goa	Pernem	Dhargal	Eggplant	2010
36	Rs-10-315	Goa	Salcete	Abajim	Eggplant	2010
37	Rs-10-319	Goa	Salcete	Chanduwada	Eggplant	2010
38	Rs-10-322	Goa	Salcete	Nuvem	Eggplant	2010
39	Rs-10-325	Goa	Quepem	Carurem	Eggplant	2010
40	Rs-10-332	Goa	Quepem	Dhavali	Chilli	2010
41	Rs-10-337	Goa	Tiswadi	Old Goa	Eggplant	2010
42	Rs-11-340	Maharashtra	Ratnagiri district	Gimvhane	Eggplant	2011
43	Rs-11-341	Maharashtra	Ratnagiri district	Wakavali	Eggplant	2011
44	Rs-11-344	Maharashtra	Ratnagiri district	Dapoli	Eggplant	2011
45	Rs-11-357	Maharashtra	Ratnagiri district	Dapoli	Eggplant	2011
46	Rs-11-359	Maharashtra	Ratnagiri district	Nigade	Eggplant	2011
47	Rs-11-364	Maharashtra	Ratnagiri district	Jalegoan	Eggplant	2011
48	Rs-11-363	Maharashtra	Ratnagiri district	Borivilli Aadawadi	Eggplant	2011
49	Rs-11-365	Maharashtra	Ratnagiri district	Gave village	Eggplant	2011
50	Rs-11-376*	Orissa	NA	Orissa	Eggplant	2011

*Isolates were procured from Culture collection of Plant Pathology Laboratory, ICAR Old-Goa for comparative study; [a]freshly wilted samples were collected from villages; NA-Not available; [#] Year of isolation.

2.2.1.2. Isolation of *R. solanacearum* from wilted samples

R. solanacearum was isolated from freshly wilted eggplant, chilli and tomato as described by Ramesh and Phadke (2012). The rootlets of the wilted sample were carefully washed twice to remove all the soil particles and adhering matter with clean tap water, air dried and kept aside for isolation. A horizontal cut was made on the rootlet

using a sterile knife or a blade, the cut-end surface from upper portion was slightly dipped in the sterile water to observe the bacterial ooze exuding from the infected xylem vascular bundles. The ooze was streaked onto sterile TZC agar plate (Casamino acid, 1g L^{-1}; peptone, 10g L^{-1}; glucose, 5g L^{-1}; agar, 17 g L^{-1} amended with 0.005 % v/v 2,3,5-triphenyl tetrazolium chloride) (Kelman, 1954) and the plates were incubated at 28$\pm$ 2°C in the incubator for 48 to 72 h. Colonies of *R. solanacearum* were identified on the basis of their fluidal nature (Wullings *et al.*, 1998) and spiral pink centers. These colonies were purified again on TZC medium. Well separated typical single virulent colonies of *R. solanacearum* were selected from TZC plates and restreaked on the CPG agar (Casamino acid, 1g L^{-1}; peptone, 10g L^{-1}; glucose, 5g L^{-1} ; and agar, 17 g L^{-1}) to obtain pure culture of *R. solanacearum*.

2.2.1.3. Preservation and storage of the pathogens

A single colony of *R. solanacearum* was inoculated in 5 mL of CPG broth and incubated at 28°C overnight on rotary shaker at 150 rpm. After 12 hours of incubation, 700 µL of the bacterial culture was taken in 1 mL cryovial to which 300 µL of glycerol was added, mixed well and stored at -80°C. Cultures were also stored at room temperature in water stocks by inoculating a single *R. solanacearum* colony into 1 mL of sterile distilled water.

2.2.2. Phenotypic characterization

2.2.2.1. Cultural and morphological characteristics

Morphological and cultural characterization of bacterial isolates on TZC medium was recorded and Gram character was determined according to standard methods described elsewhere.

2.2.2.2. Biovar determination

Biovar determination of *R. solanacearum* isolates was carried out based on utilization and oxidation of hexose alcohols and carbohydrates (Hayward, 1991). 10% solutions of the carbohydrates (Lactose, maltose, cellobiose, mannitol, dulcitol and sorbitol) were prepared and filter sterilized (0.22 µm; Millipore filters, Bangalore, India). Purple base broth (Ammonium dihydrogen phosphate, 1g L^{-1}; potassium chloride, 0.2g L^{-1}; magnesium sulfate, 0.2g L^{-1}; peptone, 1g L^{-1}; bromothymol blue, 0.3g L^{-1}) was prepared and the final pH of the medium was adjusted to 7.0-7.1 (an olivaceous green color) by addition of 40% (w/v) NaOH solution. Sterilized broth (900 µL) was dispensed into micro centrifuge tubes and carbohydrate stock (100 µL) was added (final concentration of 1.0 %) to each tube. 20µL of 24 h old grown *R. solanacearum* culture (8 log CFU mL^{-1}) was inoculated in each tube and incubated at 28±2°C. The tubes were examined at 3, 7 and 14 days for change of pH (yellow colour) from the top downwards. The test was repeated twice with two replications along with appropriate control (no carbohydrate).

2.2.2.3. PCR confirmation of *R. solanacearum* by using Rs759/760 primer set

A single, pure fluidal colony was inoculated into CPG broth and incubated overnight on rotary shaker (150 rpm) at 28±2 ˚C. The overnight grown culture was then used as template for Polymerase chain reaction. PCR was performed using Rs759/Rs760 (Rs 759 5'-GTCGCCGTCAACTCACTTTCC-3'; Rs760 5'-GTCGCCGTCAGCAATGCGGAATCG-3') primer pairs (Opina *et al.*, 1997) in a DNA Thermal Cycler (Mastercycler Pro, Eppendorf, Germany). The volume of reaction mixture was 10µL [1.0 µM of each primer, 200µM dNTPs, 1X PCR buffer, 10% DMSO, 20µg BSA, 0.5U *Taq* DNA polymerase (M/s Sigma-Adrich Chemicals) and 1µl of the

template]. PCR cycle consisted of initial denaturation at 94 °C for 3 min, annealing at 53 °C for 1 min and extension at 72 °C for 1.5 min followed by 30 cycles at 94 °C for 15 sec, 60 °C for 15 sec, 72 °C for 15 sec and a final extension at 72 °C for 5 min. The amplified product (280 bp) was separated on 1.5% agarose gel containing 0.5 µg mL^{-1} of ethidium bromide and visualized using gel documentation unit (MultiImage Light Cabinet, AlphaImager EC, Alpha Innotech Corporation, San Leandro, CA, USA).

2.2.3. Extraction of genomic DNA from *R. solanacearum*

For routine analyses, total genomic DNA was extracted from 1.5 mL CPG broth cultures grown at 28±2°C for 24 h by the following method [with modification of method suggested by Kumar *et al.* (2004)]. Bacterial culture was revived in 5 mL CPG broth from 30% glycerol stock. After 12 h of incubation, 1.5 mL of the culture suspension (OD 0.5-1.0 at 600 nm) was taken in a micro centrifuge tube and was subjected to centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was washed twice with equal volume of sterile distilled water followed by centrifugation at 10,000 rpm for 10 min at 4°C. A volume of 675µL of genomic DNA extraction buffer (Tris HCl, 100 mM; EDTA, 100 mM; Na_2HPO_4, 100 mM; NaCl, 1.5 M; CTAB, 1%; Proteinase K, 20µg; Lysozyme, 100µg) was added to the pellet in the micro centrifuge tube followed by incubation at 37°C for 30 min in an rotary evaporator (Strike 102, Steroglass Rotary Evaporator Instruments, and Kentron). The mixture was vortexed after every 10 min. To this mixture, 75µL of 20% SDS (Appendix C.1) was added and then incubated at 65°C for 2 h with intermittent vortexing at every 10 min. The tubes were then centrifuged at 11,000 rpm for 10 min at 4°C. The clear solution obtained was collected in a new micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. The

mixture was mixed thoroughly and again centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a new micro centrifuge tube, 0.6 volume of isopropyl alcohol was added and the mixture was incubated at room temperature for 60 min. The tubes were centrifuged again at 10,000 rpm for 10 min at room temperature. The pellet was later washed twice with 500µL of 70% ethanol (Appendix C.1) by centrifuging at 10,000 rpm for 10 min at room temperature. The pellet was dried and then dissolved in 50µL sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 20µg mL^{-1} of RNase and stored at -20°C.

2.2.3.1. Agarose gel electrophoresis of DNA

Electrophoresis was performed using 1X TAE buffer (Tris HCl, 4.8g L^{-1}; 0.5M EDTA, 2.0 mL; glacial acetic acid, 1.14 mL) as electrode buffer at constant voltage of 80 V. Agarose (0.8%) (Appendix C.4) was prepared in 1X TAE buffer. Ethidium bromide (0.5 µg mL^{-1}) was added to the melted agarose. DNA samples (5µL) containing genomic DNA were mixed with 1µL of 6X DNA loading buffer (Appendix C.3), mixed using pipette and loaded into the wells of agarose gel flooded with 1X TAE buffer. The electrophoresis was done usually until the dye front travelled 2/3rd of the agarose gel. The gel was viewed and photographed using gel documentation unit (MultiImage Light Cabinet, AlphaImager EC, Alpha Innotech Corporation, San Leandro, CA, USA).

2.2.3.2. Quantification of genomic DNA

The genomic DNA obtained was further quantified using Nano drop-1000, Thermo fisher scientific, USA as per the manufacturer's instructions. DNA was diluted to final concentration of 50ng μL^{-1} and used as template for all the PCR reactions.

2.2.4. Phylotype-specific-multiplex PCR amplification

The phylotype assignment of the isolates was carried out by a Pmx-PCR (Phylotype multiplex PCR) as described by Fegan and Prior (2005) with four forward primers (Table 2 B) each specific for one phylotype and one reverse primer.

Table 2 B. Details of Primers used for phylotype determination in the present study

Primer Specificity	**Primers**	**Expected Size (bp)**
Forward primer for Phylotype 1	Nmult21:1F(5'-CGTTGATGAGGCGCGCAATTT-3')	144 bp
Forward primer for Phylotype 2	Nmult21:2F(5'-AAGTTATGGACGGTGGAAGTC-3')	372 bp
Forward primer for Phylotype 3	Nmult23:AF(5'ATTACSAGAGCAATCGAAAGATT -3')	91 bp
Forward primer for Phylotype 4	Nmult22:InF(5'-ATTGCCAAGACGAGAGAAGTA -3')	213 bp
Reverse primer for all Phylotype	Numult22:RR(5'TCGCTTGACCCTATAACGAGTA -3')	NA
Species Specific forward primer	Rs759 (5'-GTCGCCGTCAACTCACTTTCC-3'),	
Species Specific forward primer	Rs760 (5'-GTCGCCGTCAGCAATGCGGAATCG-3')	280 bp

Reaction mixture (25µL) consisted of 1 X *Taq* Buffer (10mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin), 200µM of each dNTP, 6 pmol each of Nmult21:1F, Nmult21:2F, Nmult22: InF primers, 18pmol of Nmult23: AF, 10 pmol of Nmult22: RR and 4 pmol each of Rs759 and Rs760, 2 units of *Taq* DNA polymerase, 50ng of DNA template. PCR was performed in Mastercycler Pro (Eppendorf, GmBH) using the following cycle: initial denaturation of 96 °C for 5 min; 30 cycles of 94 °C for 15 sec, 59 °C for 30 sec, 72 °C for 30 sec and final extension of 10 min at 72 °C. 5µL of the PCR product was separated on 2 % agarose gel containing 0.5 µg mL^{-1} of ethidium bromide at 80 V for 1 h. Gels were documented using the MultiImage Light Cabinet (Alpha Innotech Corporation). DNA of phylotypes I, II, III and IV respectively were provided by C. Allen (University of Wisconsin-Madison, USA).

2.2.5. Pathogenicity and host range studies

Eggplant, tomato and chilli are grown on larger scale in Goa and therefore the pathogenicity of all the isolates was assessed using these hosts. Seeds of eggplant (*Solanum melongena* L.) cultivar *Agassaim* variety, chilli (*Capsicum annum* L.) variety *Pusa jwala* and tomato (*Lycopersicum esculentum* L.) variety *Arka vikas* were sown in nursery mixture (Soil: FYM: sand at the ratio of 2:1:1) and maintained in a greenhouse at 30°C. Twenty-day old seedlings were transplanted into plastic pots containing sterile nursery mixture and watered every alternate day in the greenhouse. Simultaneously, the *R. solanacearum* isolates were grown in 20 mL CPG broth for 48 h and the culture was centrifuged at 10,000 rpm for 8 min at 28°C and the pellet was suspended in 20 mL of sterile 1X PBS (NaCl, 8g L^{-1}; KCl, 0.2g L^{-1}; $Na_2HPO_4.2H_2O$, 1.44g L^{-1} and KH_2PO_4, 0.24g L^{-1}; pH 7.4) (Huang and Allen, 1997). Soil from 6 cm around the eggplant rhizosphere region was loosened for better aeration and 10 mL of inoculum (8 log CFU mL^{-1}) was poured per plant by soil drenching method (Schaad *et al.*, 2001). Five plants were inoculated per replication. Plants inoculated with sterile 1X PBS served as negative control. Prior to inoculation, plants were not watered for 24 h (Williamson *et al.*, 2002). The disease incidence and percentage of wilt were recorded every alternate day for about 28 to 30 days.

2.2.6. Biochemical tests

Based on the pathogenicity results a single highly virulent isolate was selected and was used to perform a few biochemical tests viz. potassium hydroxide solubility test, catalase test, gelatin hydrolysis, oxidase test, growth at 37°C and 41°C, production of fluorescent pigmentation and nitrate reduction test as per standard protocols. Two isolates Rs-09-100

and Rs-09-109 were also taken from the Culture collection of Plant Pathology Laboratory, ICAR, Research Complex for Goa, Goa for comparative analysis. These 3 isolates were further used in all the studies as representative isolates from Goa.

The biochemical test protocols are as follows:

2.2.6.1. Potassium hydroxide solubility test: 2-3 colonies of *R. solanacearum* were picked using wire loop and mixed rigorously with few drops of 3 % KOH solution on a glass slide for 10 sec. The test was recorded as positive when bacterial solution got sticky forming slime threads with wire loop (Suslow *et al.*, 1982).

2.2.6.2. Catalase test: Few colonies of fresh *R. solanacearum* culture were mixed with 2-3 drops of hydrogen peroxide solution on a glass slide. Production of gas bubbles was recorded as positive reaction (Schaad, 1980).

2.2.6.3. Gelatin hydrolysis: *R. solanacearum* isolates were inoculated in nutrient gelatin medium (Peptone, 5g L^{-1}; beef extract, 3g L^{-1}; gelatin, 20g L^{-1}; pH 7.0) and incubated at room temperature for 24 h, later the tubes were refrigerated for 30 min and the medium were observed. Liquid medium after refrigeration showed positive test.

2.2.6.4. Oxidase test: A loop full of fresh bacterial culture was smeared on the moist filter paper soaked with few drops of Tetramethyl paraphynylene diamine (TMPD) dye. Production of deep purple colour in 5-10 sec indicated a positive oxidase test. This dye act as electron accepter and get reduced.

2.2.6.5. Nitrate reduction test: Nitrate broth (Peptone, 5g L^{-1}; beef extract, 3g L^{-1}; KNO_3, 1g L^{-1}; NaCl, 5g L^{-1}; pH 7.0) was inoculated with culture and incubated at room temperature for 24 h. After incubation, 5 drops of sulphanilic acid and 5 drops of α-

naphthylamine were added. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5 mg of zinc.

2.2.6.6. Growth at 37°C and 41°C: To determine the growth of isolates at 37°C and 41°C, the isolates were streaked on CPG agar and incubated at the desired temperature for 48 h.

2.2.6.7. Pigment production: *R. solanacearum* was streaked on King's B Medium ($MgSO_4.7H_2O$, 1.5g L^{-1}; KH_2PO_4, 1.5g L^{-1}; proteose peptone, 10g L1; glycerol, 10 mL L^{-1}, bacteriological agar, 15g L^{-1}) and incubated at 30°C for two days and observed for brown pigmentation and fluorescence was observed under UV lamp (King *et al.*, 1954).

2.2.7. Genetic characterization of *R. solanacearum* isolates

Genetic diversity of *R. solanacearum* was analyzed by using various molecular methods such as ARDRA, PCR-RFLP, rep-PCR and sequencing studies.

Table 2 C. Details of the isolates used in genetic characterization of *R. solanacearum*

No.	Method	Number of isolates under study	Number of Reference isolates
1	ARDRA	5[a]	4[b]
2	PCR-RFLP analysis of *fliC* gene	5[a]	4[b]
3	PCR-RFLP analysis of 280 bp fragment	5[a]	4[b]
4	rep-PCR (ERIC and BOX)	50	Refer Table 2 A
5	Gene sequencing	10	Refer Table 2 G
6	MLST analysis	10	Refer Table 2 H

[a]*R. solanacearum* isolates from different geographical locations and host were selected from India (Rs-08-17, Rs-09-113, Rs-09-197, Rs-10-209, Rs-11-365); A few of these isolates were procured from Culture collection of Plant pathology Laboratory, ICAR, Goa; [b]DNA template was used from GMI1000 (Phylotype-I), K60 (Phylotype-II), WW386 (Phylotype-III) and WW443 (Phylotype-IV).

2.2.7.1. Polymerase Chain Reaction- Restriction Fragment Length Polymorphism analyses (PCR-RFLP)

2.2.7.1.1. 16S rRNA (Amplified Ribosomal DNA Restriction Analysis-ARDRA)

16S rRNA (~1500 bp) gene was amplified from 5 *R. solanacearum* isolates (Table 2 C) from this study and reference isolates (Phylotype I, II, III and IV) using 16F-27 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16R-1492 (5'-GGTTACCTTGTTACGACTT-3') (Jeong *et al.*, 2007) primers. For 16S rRNA PCR, 20µL reaction mix containing 1X PCR buffer, 200µM dNTPs, 0.4µM of primers- 16F-27 and 16SR-1492 each, 1.0 U *Taq* polymerase and 50 ng template DNA was used. PCR cycle consisted of initial denaturation at 94°C for 5 min followed by 32 cycles at 94°C for 30 sec, 55°C for 40 sec and 72°C for 1 min followed by a final extension period at 72°C for 5 min. The gene was amplified according to the manufacturer's instructions and later digested with restriction endonucleases (1 U of *Alu* I and *Msp* I each; Fermentas Life Sciences).

2.2.7.1.2. *fliC* gene

fliC gene (380 bp) was amplified from *R. solanacearum* isolates (Table 2 C) and reference isolates (Phylotype I, II, III and IV) using Rsol_*fliC* forward (5'-GAACGCCAACGGTGCGAACT-3') and Rsol_*fliC* reverse (5'-GGCGGCCTTCAGGGAGGTC-3') primers (Schonfeld *et al.*, 2003). Reaction mix (25µL) contained 1X PCR buffer, 200 µM dNTPs, 10 pM of primers- Rsol_*fliC* forward Rsol_*fliC* reverse each, 10% DMSO, 2.0 U *Taq* polymerase and 100ng template DNA. The PCR cycle was set as: initial denaturation at 94°C for 5 min and then cycled through 25 cycles of 94°C for 30 sec, 63°C for 2 min and 72°C for 1 min followed by a final extension period at 72°C for 10 min. The amplified *fliC* gene fragments were digested

with 1 U of *Alu* I restriction endonuclease enzyme (Fermentas Life Sciences) according to the manufacturer's recommendations.

2.2.7.1.3. 280 bp fragment

280bp gene fragment (a part of *lpxC* gene for UDP-3-*O*-acyl *N*-acetylglucosamine deacetylase fragment) was amplified from *R. solanacearum* isolates (Table 2 C) and reference isolates (Phylotype I, II, III and IV) using Rs759/760 primers as described in the *R. solanacearum* PCR confirmation section (2.2.2.3). 280 bp amplicon was digested with 1 U of *Nla* III restriction endonuclease (New England Biolabs Inc., Beverly, MA).

All the above PCR amplifications were carried out in DNA Thermal Cycler (Mastercycler Pro, Eppendorf, Germany). Restriction digestion reaction consisted of 100-150ng DNA, 1X tango buffer and 1U of restriction enzyme incubated at 37 °C for 1 h. The restricted fragments were separated on 2% agarose with ethidium bromide at 50 V and the gel was visualized using gel documentation unit (MultiImage Light Cabinet, AlphaImager EC, Alpha Innotech Corporation, San Leandro, CA, USA).

2.2.7.2. Rep-PCR analysis

The repetitive sequence based PCR fingerprint method was used for genetic diversity analysis (Xu *et al.*, 2010). In this study, BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') and ERIC primers (ERIC 1R 5'-ATGTAAGCTCCTGGGGATTCAA-3 and ERIC2 5'-AAGTAAGTGACTGGGGTGAGCG-3') synthesized at M/S Sigma Aldrich, Bangalore were used.

PCR amplification was carried out in DNA Thermal Cycler (Mastercycler Pro, Eppendorf, Germany). 25µL reaction mix contained 5X Gitschier's buffer [83 mM

$(NH_4)_2SO_4$, 33.5mM $MgCl_2$, 335mM Tris-HCl (pH 8.8), 33.5µM EDTA, 150mM β-merceptoethanol and distilled water], 10% DMSO, 10µg BSA, 250µM each dNTPs, primers (50µM of each ERIC primers for ERIC PCR; 30µM of BOX AIR primer for BOX PCR), 2.5U of DNA polymerase *Taq* (Sigma) and 100ng of template DNA. The following PCR cycle parameters were set: an initial denaturation of 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 65°C for 8 min with a final extension step of 65°C for 15 min. Amplified PCR products (25µL) were separated by 2.0 % agarose gel electrophoresis in 1X TAE buffer at 40 V cm^{-1} for 6 h, stained with ethidium bromide, and the gel was visualized using gel documentation unit. For confirmation of the banding patterns, DNA was re-extracted and PCR were repeated twice for each isolate.

2.2.7.3. Analysis of PCR-RFLP and Rep-PCR data

The PCR fingerprint profiles were used to measure genetic similarity among isolates. The various bands obtained from ARDRA, PCR-RFLP, BOX and ERIC PCR were compared and scored as 1 for the presence of band and 0 for absence of a band based on different electrophoretic mobility. The variations in the intensity of band were not considered as differences. The final binary data were analyzed using NTSys software v 2.02i (Applied Biostatistics Inc. USA) and the dendrogram was constructed from the similarity coefficient data by the unweighted pair group method with arithmetic averages clustering (Sneath and Sokal, 1973) under SAHN (sequential, agglomerative, hierarchical, nested clustering) module of NTSYSpc. The similarity coefficients for all possible pairs of isolates based on the fingerprint groups was estimated by the Dice method (Dice, 1945).

75% similarity coefficient was used to define BOX and ERIC fingerprints (Xue *et al.*, 2011).

2.2.7.4. Sequencing of genes

2.2.7.4.1. Amplification of genes: Seven different genes (*egl*, *adk*, *fliC*, *mutS*, *ppsA*, *gapA*, *pglA*) were selected in this study based on the previous phylogenetic reports and the reference studies on *R. solanacearum* (Castillo and Greenberg, 2007; Wicker *et al.*, 2012). Ten isolates were chosen based on the results of BOX PCR fingerprints, host and geographical origin of the isolates (Table 2 D).

Table 2 D. List of *R. solanacearum* isolates selected for sequencing studies based on the host, geographical location and BOX PCR analysis

No.	Isolate	State	Host	BOX-PCR Cluster	Total number of isolates statewise
1	Rs-08-44	Goa	Eggplant	I	
2	Rs-09-172	Goa	Eggplant	III	
3	Rs-10-278	Goa	Eggplant	V	
4	Rs-10-286	Goa	Eggplant	VII	7
5	Rs-10-325	Goa	Eggplant	IX	
6	Rs-10-332	Goa	chilli	I	
7	Rs-09-83	Goa	Tomato	V	
8	Rs-11-344	Maharashtra	Eggplant	IV	2
9	Rs-11-363	Maharashtra	Eggplant	VI	
10	Rs-11-376	Orissa	Eggplant	V	1

Three chromosomal housekeeping genes (*ppsA*, phosphoenolpyruvate synthase; *adk*, adenylate kinase and *gapA*, glyceraldehyde 3-phosphate dehydrogenase oxidoreductase) and two megaplasmid virulence-related genes (*fliC*, encoding flagellin protein; and *egl*, endoglucanase) were selected. In addition to these genes, *mutS* (DNA repair gene) and *pglA* (Polygalacturonase gene) were also included in the study. Details of the primer sequences previously described and their amplicon sizes are given in Table 2 E.

Table 2 E. Details of primers used to amplify internal fragments of selected genes

No.	Gene	Product size (bp)	Primer designation[a]	Sequences	References
1	*gapA*	980	gapA-F[a] gapA-R[b]	5'-ATGACCATCAAGATCGGCAT3' 5'-GGGCCATTTCCAGCACCT-3'	Castillo and Greenberg, 2007
2	*adk*	536	adk F[a] adk R[b]	5'-TCTGTTGGGCGCACCCGGC-3' 5'-CCCAGCCGGAGTAGTAGTCC-3'	Castillo and Greenberg, 2007
3	*fliC*	380	Rsol *fliC*-F[a] Rsol *fliC*-R[b]	5'-GAACGCCAACGGTGCGAACT-3' 5'-GGCGGCCTTCAGGGAGGTC-3'	Schönfeld *et al.*, 2003
4	*ppsA*	1210	ppsAN-F[a] ppsAN-R[b]	5'-GGGCGTGATGTTCACGAT-3' 5'-CCAGCATGGGGTTCTCTTC-3'	Castillo and Greenberg, 2007
5	*mutS*	758	mutS-Rs-F[a]-1570 mutS-Rs-R[b]-1926	5'-ACA GCG CCT TGA GCC GGTACA-3' 5'-GCT GAT CAC CGG CCC GAA CAT-3'	Prior and Fegan, 2005
6	*egl*	750	Endo-F[a] Endo-R[b]	5'-ATGCATGCCGCTGGTCGCCGC-3' 5'-GCGTTGCCCGGCACGAACACC-3'	Ivey *et al.*, 2007
7	*pglA*	504	Peh F[a] Peh R[b]	5'-CAGCAGAACCCGCGCCTGATCCAG-3 5'-ATCGGACTTGATGCGCAGGCCGTT-3'	Gillings *et al.*, 1993

[a]F, forward primer; [b]R, reverse primer; The same primers were also used as sequencing primers.

All the genes were amplified using different reaction mixtures and conditions as mentioned in Table 2 F.

Table 2 F. PCR conditions for gene amplification

Gene	Amplicon size (bp)	PCR reaction mixture	PCR conditions
gapA gene	980	50µL Reaction mixture containing 1X PCR buffer, 200µM dNTPs, 10pM of primers gapA-F and gapA-R each, 6% DMSO, 1U *Taq* polymerase and 100ng template DNA	Initial denaturation at 95°C for 9 min and then cycled through 35 cycles of 95°C for 30 sec, 55°C for 60 sec and 72°C for 2 min followed by a final extension period at 72°C for 10 min
adk gene	536	50µL Reaction mix containing 1X PCR buffer, 200µM dNTPs, 10pM of primers- adk-F and adk-R each, 25mM $MgCl_2$, 6% DMSO, 1U *Taq* polymerase and 100ng template DNA	Initial denaturation at 95°C for 9 min and then cycled through 35 cycles of 95°C for 30 sec, 62°C for 60 sec and 72°C for 60 sec followed by a final extension period at 72°C for 10 min

Gene	Amplicon size (bp)	PCR reaction mixture	PCR conditions
fliC gene	380	25µL Reaction mix containing 1X PCR buffer, 200µM dNTPs, 10pM of primers- Rsol_*fliC* forward Rsol_*fliC* reverse each, 10% DMSO, 2.0 U *Taq* polymerase and 100ng template DNA	Initial denaturation at 94°C for 5 min and then cycled through 25 cycles of 94°C for 30 sec, 63°C for 2 min and 72°C for 1 min followed by a final extension period at 72°C for 10 min
ppsA gene	1210	50µL Reaction mix containing 1X PCR buffer, 200µM dNTPs , 10pM of ppsA-NF and ppsA-NR primers each, 6% DMSO and 1U *Taq* polymerase and 100ng template DNA	Initial denaturation at 95°C for 9 min followed by 35 cycles for 30 sec at 95°C, 1 min at 57°C and 2 min for 72°C followed by a final extension period at 72°C for 20 min
mutS gene	758	50µL Reaction mix containing 1X PCR buffer, 200µM dNTPs, 0.25µM of primers- mutS-Rs-F-1570 and mutS-Rs-R-1926 each, 5% DMSO, 1.25 U *Taq* polymerase and 100ng template DNA	Initial denaturation at 96°C for 5 min and then cycled through 35 cycles of 94°C for 60 sec, 66°C for 60 sec and 72°C for 60 sec followed by a final extension period at 72°C for 10 min
egl gene	750	100 ng template DNA 1X PCR buffer, 200µM dNTPs , 0.25µM of primers- Endo-F and Endo-R each, 5% DMSO, 1 U *Taq* polymerase and 100ng template DNA	Initial denaturation at 96°C for 9 min followed by 30 cycles at 95°C for 1 min, 70°C for 1 min and 72°C for 2 min followed by a final extension period at 72°C for 10 min.
pglA gene	504	50µL containing 1X PCR buffer, 200µM dNTPs, 0.25 µM of PehF and PehR primers each, 5% DMSO, 1U *Taq* polymerase and 100ng template DNA	Initial denaturation at 96°C for 1 min followed by 2 cycles of 96°C for 30 sec, 70°C for 30 sec and 72°C for 1 min and 33 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 5 min.

All the PCR gene amplifications mentioned above were carried out in Eppendorf Mastercycler vapoprotect. PCR products were examined by electrophoresis on 1% agarose in TAE buffer followed by purification using GeneJET™ PCR Purification Kit (Fermentas Life Sciences, EU) according to the manufacturer's Instruction. The purified amplicons were sequenced and further analyzed.

2.2.7.4.2. Gene sequencing: All the gene products were sequenced using ABI 3500xL Genetic Analyzer; sequencing reaction was performed using Big Dye Terminator version

3.1” Cycle sequencing kit using forward and reverse primers used for amplification of the respective genes.

2.2.7.4.3. Phylogenetic analysis: Raw sequences from both strands were manually aligned using vector NTI advance 11.5.2 software (Invitrogen) and trimmed.Sequences of other reference isolates (Table 2 G) were obtained from NCBI for comparative sequence data analysis of isolates under this study for their evolutionary relationships by constructing phylogenetic trees. Genetic distance data was employed for constructing the phylogenetic trees by using the neighbor-joining (NJ using Jukes and Cantor alogorithm) method (Saitou and Nei, 1987; Jukes and Cantor, 1969) with 1000 bootstrap resampling of the data.

Table 2 G. Reference sequences of *R. solanacearum* obtained from the Genbank database for gene sequence comparison

No.	Isolate	Host of isolation	Country	Year	Phylotype	Sequevar
1	GMI1000	*Solanum lycopersicum*	French Guiana	1978	I	18
2	GMI8254	*S. lycopersicum*	Indonesia	NA	I	47
3	CIP365	*Solanum tuberosum*	Philippines	1989	I	45
4	PSS004	*S. lycopersicum*	Taiwan	1988	I	15
5	PSS219	*S. lycopersicum*	Taiwan	1996	I	34
6	R292	*Morus alba*	China	NA	I	12
7	MAD-017	*Capsicum annuum*	Madagascar	2006	I	46
8	Rs-08-11	*S. melnogena*	India	2008	I	unknown
9	Rs-08-55	*S. melnogena*	India	2008	I	unknown
10	Rs-09-94	*C. annum*	India	2009	I	unknown
11	Rs-10-216	*Zingiber officinale*	India	2010	I	17
12	Rs-10-250	*S. melnogena*	India	2010	I	14
13	K60	*S. lycopersicum*	USA	1953	IIA	7
14	CFBP6784	*Anthurium andreanum*	Martinique	1999	IIB	4
15	UW588	*Musa acuminata*	Guatemala	2004	IIA	6
16	UW070	*Banana Plantain*	Colombia	1961	IIB	4
17	CFBP2957	*S. lycopersicum*	Martinique	1987	IIA	36
18	IPO1609	*S. tuberosum*	Netherlands	NA	IIB	1
19	MOLK2	*Musa sp.*	Philippines	1991	IIB	3
20	JT516	*S. tuberosum*	Reunion island	1993	IIB	1
21	CIP240	*S. tuberosum*	Brazil	1983	IIB	26
22	NCPPB0332	*S. tuberosum*	Zimbabwe	1954	III	21
23	J25	*S. tuberosum*	Kenya	1998	III	20
24	UW034	*Nicotiana tabacum*	Zimbabwe	1959	III	NA
25	MAD-029	*S. tuberosum*	Madagascar	2006	III	19
26	NCPPB1018	*S. tuberosum*	Angola	1961	III	22

No.	Isolate	Host of isolation	Country	Year	Phylotype	Sequevar
27	DGBBC1138	*S. tuberosum*	Guinea	NA	III	44
28	MAFF301558	*S. tuberosum*	Japan	NA	IV	8
29	R028	*Syzygium aromaticum*	Indonesia	NA	IV	9
30	UW521	*S. aromaticum*	Indonesia	1987	IV	NA
31	R229	*M. acuminata*	Indonesia	1988	IV	10
32	PSI07	*S. lycopersicum*	Indonesia	NA	IV	10
33	R230	*Musa sp.*	Indonesia	1998	IV	10
34	UW519	*S. aromaticum*	Indonesia	1978	IV	NA

NA- Not available

2.2.7.5. Multiple Locus Sequence Typing (MLST) of *R. solanacearum* isolates

Sequences for MLST analysis were aligned using the Clustal W alignment tool (Thompson *et al.*, 1994) available in MEGA 5.0 software (Tamura *et al.*, 2011), along with reference sequences from the complete genomes (Table 2 H).

Table 2 H. Reference sequences of *R. solanacearum* obtained from the Genbank database for MLST analysis.

No.	Isolate	Host of isolation	Country	Year	Phylotype	Sequevar[a]
1	GMI1000	*S. lycopersicum*	French Guiana	1978	I	18
2	GMI8254	*S. lycopersicum*	Indonesia	NA	I	47
3	CIP365	*S. tuberosum*	Philippines	1989	I	45
4	PSS219	*S. lycopersicum*	Taiwan	1996	I	34
5	R292	*Morus alba*	China	NA	I	12
6	UW558	*S. lycopersicum*	Monjas Jalapa	NA	I	14
7	Rs-09-161	*S. melongena*	India	2009	I	Unknown
8	Rs-10-244	*C. annuam*	India	2010	I	48

[a]Sequevars are clusters of strains whose *egl* partial sequences differ by less than 1% (Fegan and Prior, 2005); NA- Not available

All the phylogenetic related indices and parameters along with the haplotypes were determined using DNASP 5.0 (Librado and Rozas, 2009). The genes were concatenated based on the gene alignment on GMI1000 Phylotype I isolate. Neutrality tests viz. Tajima's D, Fu and Li's D and F tests were calculated to determine the significance for each gene separately, for the concatenated set of these genes and on each phylotype set too. In a classic neutrality test, the null hypothesis of neutral evolution corresponds to D

= 0. Departures from neutrality may be indicated by significant deviations of D from 0. For example, D > 0 may be a result of positive (balancing or diversifying) selection or shrinking populations; D > 2 were considered as significant (Tajima, 1989). Splitstree4 was used to draw a phylogenetic construct on the total concatenated sequence data of 10 isolates along with the reference isolates using the Neighbor-net algorithm.

2.2.7.6. Nucleotide sequence accession numbers

The nucleotide sequence data of the 10 representative *R. solanacearum* isolates have been deposited into the GenBank database and the accession numbers were obtained.

2.3. Results and Discussion

2.3.1. Isolation and preservation of *R. solanacearum* isolates

Bacterial wilt samples were collected from major vegetable growing areas of Goa (Fig. 2.1) from different crops (eggplant, chilli and tomato) during the period 2008 to 2011 (Table 2 A).

Fig. 2.1 Map of Goa showing major vegetable growing areas used for sample collection of wilted Solanaceous crops. Numbers of isolates isolated from the each taluka from Goa are presented in brackets.

Table 2 A. List of *R. solanacearum* isolates used in this study

No	Isolate	State	Taluka	Place of isolation[a]	Host	Year[#]
1	Rs-08-01	Goa	Tiswadi	Pilar	Eggplant	2008
2	Rs-08-17	Goa	Tiswadi	Sulabhat	Eggplant	2008
3	Rs-08-43	Goa	Tiswadi	Caranzalem	Eggplant	2008
4	Rs-08-44	Goa	Tiswadi	Mansher	Eggplant	2008
5	Rs-08-47	Goa	Tiswadi	Mansher	Eggplant	2008
6	Rs-08-49	Goa	Tiswadi	Caranzalem	Eggplant	2008
7	Rs-08-61	Goa	Tiswadi	Caranzalem	Eggplant	2008
8	Rs-08-74	Goa	Tiswadi	Taligoa	Eggplant	2008
9	Rs-09-83	Goa	Pernem	Kasar	Tomato	2009
10	Rs-09-84	Goa	Tiswadi	Caranzalem	Eggplant	2009
11	Rs-09-86	Goa	Tiswadi	Caranzalem	Eggplant	2009
12	Rs-09-104	Goa	Tiswadi	Taligoa	Eggplant	2009
13	Rs-09-113*	Kerala	Trichur	Vettingapadam	Chilli	2009
14	Rs-09-123*	Kerala	Kottapalum	Kottapalum	Eggplant	2009
15	Rs-09-153	Goa	Canacona	Upta	Eggplant	2009
16	Rs-09-157	Goa	Mormugoa	Mestawada	Eggplant	2009
17	Rs-09-172	Goa	Tiswadi	Malawara	Eggplant	2009
18	Rs-09-173	Goa	Tiswadi	Malawara	Eggplant	2009
19	Rs-09-176	Goa	Tiswadi	Malawara	Eggplant	2009
20	Rs-09-189*	Karnataka	Hassan	Salagame Hobli	Potato	2009
21	Rs-09-197*	Karnataka	Mysore	Udduru	Chilli	2009
22	Rs-09-201*	Karnataka	Chikballapur	Nagalmuddama	Chilli	2009
23	Rs-10-209*	Kerala	Thrissur	Pachikere	Tomato	2010
24	Rs-10-216*	Kerala	NA	Calicut	Ginger	2010
25	Rs-10-250*	Andaman	NA	Andaman	Eggplant	2010
26	Rs-10-262	Goa	Tiswadi	Mansher	Eggplant	2010
27	Rs-10-266	Goa	Tiswadi	Nagali	Eggplant	2010
28	Rs-10-268	Goa	Tiswadi	Saplaan	Eggplant	2010
29	Rs-10-273	Goa	Tiswadi	Taligoa	Eggplant	2010
30	Rs-10-277	Goa	Tiswadi	Mandur	Eggplant	2010
31	Rs-10-278	Goa	Tiswadi	Pilar	Eggplant	2010
32	Rs-10-280	Goa	Ponda	Keri	Chilli	2010
33	Rs-10-286	Goa	Bicholim	Amona	Eggplant	2010
34	Rs-10-295	Goa	Bardez	Guirim	Eggplant	2010
35	Rs-10-298	Goa	Pernem	Dhargal	Eggplant	2010
36	Rs-10-315	Goa	Salcete	Abajim	Eggplant	2010
37	Rs-10-319	Goa	Salcete	Chanduwada	Eggplant	2010
38	Rs-10-322	Goa	Salcete	Nuvem	Eggplant	2010
39	Rs-10-325	Goa	Quepem	Carurem	Eggplant	2010
40	Rs-10-332	Goa	Quepem	Dhavali	Chilli	2010
41	Rs-10-337	Goa	Tiswadi	Old Goa	Eggplant	2010
42	Rs-11-340	Maharashtra	Ratnagiri district	Gimvhane	Eggplant	2011
43	Rs-11-341	Maharashtra	Ratnagiri district	Wakavali	Eggplant	2011
44	Rs-11-344	Maharashtra	Ratnagiri district	Dapoli	Eggplant	2011
45	Rs-11-357	Maharashtra	Ratnagiri district	Dapoli	Eggplant	2011
46	Rs-11-359	Maharashtra	Ratnagiri district	Nigade	Eggplant	2011
47	Rs-11-364	Maharashtra	Ratnagiri district	Jalegoan	Eggplant	2011

No	Isolate	State	Taluka	Place of isolation[a]	Host	Year[#]
48	Rs-11-363	Maharashtra	Ratnagiri district	Borivilli Aadawadi	Eggplant	2011
49	Rs-11-365	Maharashtra	Ratnagiri district	Gave village	Eggplant	2011
50	Rs-11-376*	Orissa	NA	Orissa	Eggplant	2011

*Isolates were procured from Culture collection of Plant Pathology Laboratory, ICAR Old-Goa for comparative study; [a]freshly wilted samples were collected from villages; NA-Not available; [#]Year of isolation.

The pathogen isolated on CPG medium amended with tetrazolium chloride appeared as fluidal white irregular virulent colonies of *R. solanacearum* (Fig. 2.2 a) with pink centers (Fig. 2.2 b) and produced abundant extracellular polysaccharide (EPS) (Fig. 2.2 c) on the CPG medium. The colonies exhibited a typical spiral whirling pattern and formazan pigmentation after 48 h of incubation (Fig. 2.2 d). All the virulent *R. solanacearum* from Goa and other states of India morphologically resembled those from other regions of the world (He *et al.*, 1983; Williamson *et al.*, 2002; Hayward, 1964). Overall, thirty three isolates were isolated from Goa and eight from Maharashtra.

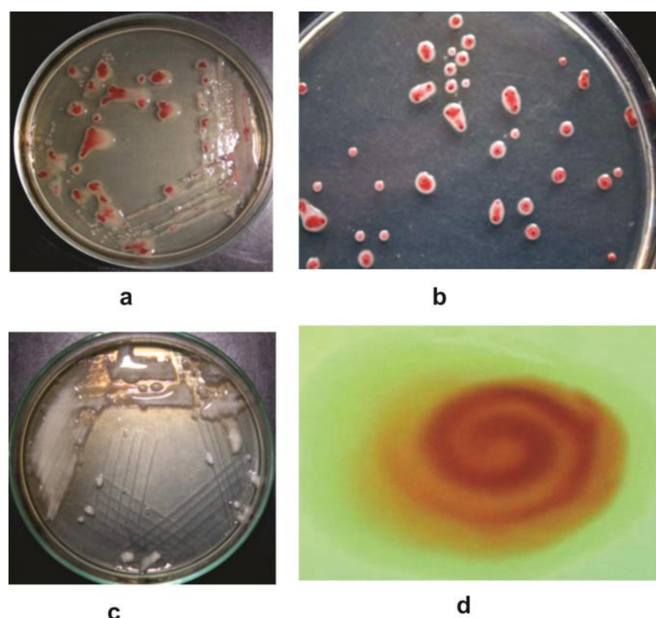


Fig. 2.2 Phenotypic and cultural characters of *R. solanacearum* on (a) CPG agar amended with 2, 3, 5-triphenyl tetrazolium chloride (TZC), (b) typical *R. solanacearum* colonies on TZC medium, (c) EPS production on CPG medium (d) typical *R. solanacearum* colony with spiral pink center after 48 h of incubation at 30°C. The pink center is due to conversion of TZC into insoluble formazon.

Nine isolates were obtained from Plant Pathology laboratory, ICAR, Research Complex for Goa, Goa (Table 2 A; Fig. 2.3). The cultures were preserved and stored in 30% glycerol stocks at -80 °C.

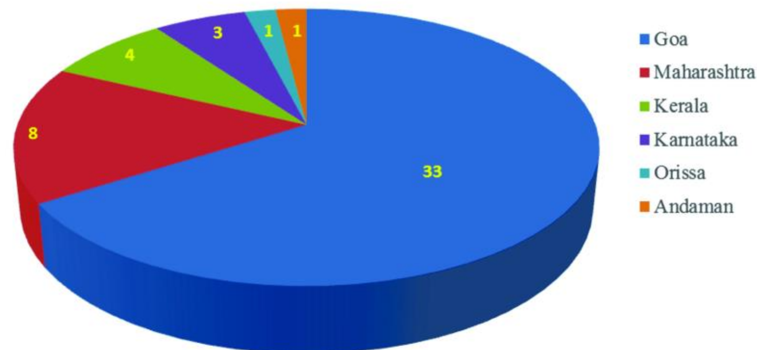


Fig. 2.3 Number of *R. solanacearum* isolates from different states of India under the present study

2.3.2. Phenotypic characterization

2.3.2.1. Cultural and morphological characteristics

Morphological studies showed that bacterial isolates were Gram negative, rod shaped and non-capsulated measuring in the range of 80-110 µm (Fig. 2.4 a).

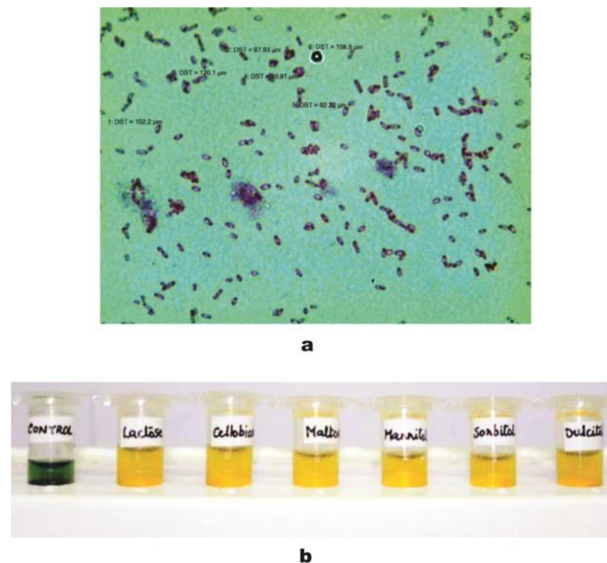


Fig. 2.4 (a) Gram staining of *R. solanacearum* showing Gram negative bacteria, (b) Biovar determination tests based on carbohydrate oxidation of *R. solanacearum* isolates. Yellow color = acid production, green color =no acid production.

2.3.2.2. Biovar determination

In this study, results for biovar characterization indicated that all the isolates utilized 6 sugars and therefore belong to biovar 3 (Fig. 2.4 b). Kumar *et al.* (2013) has also reported the presence and existence of biovar 3 isolates from India infecting solanaceous crops. Amongst the 50 isolates, one isolate (Rs-09-113) from Kerala was grouped under the new biovar 6 due to non-utilization of dulcitol as designated by Xue *et al.* (2011). Similarly, James *et al.* (2003) also reported the variation in dulcitol utilization of isolates from Kerala. Shekhawat *et al.* (1978) reported that biovar 3 and 4 were encountered only among eastern parts of India.

All the results were further confirmed with the earlier reports by Hayward (2000) who reported that Asian-origin Race1/Biovar3/Phylotype I strains of *R. solanacearum* are known to affect several important solanaceaous vegetables in tropical countries.

2.3.2.3. PCR confirmation of *R. solanacearum* by Rs759/760 primer set

In PCR analysis using Rs759/760 primer sets, all the isolates amplified a single 280 bp fragment as reported by Opina *et al.* (1997) which confirmed the identity of *R. solanacearum* (Fig. 2.5 b). This gene fragment is ubiquitous and is a part of the putative *lpxC* gene for UDP-3-*O*-acyl *N*-acetylglucosamine deacetylase in whole genome of the pathogen and can be easily amplified (Villa *et al.*, 2003). This report is in conformity with the previous studies (Ramesh *et al.*, 2014b; Chandrashekara *et al.*, 2012; Seal *et al.*, 1993).

2.3.3. Genomic DNA from *R. solanacearum*

Genomic DNA (Fig. 2.5 a) isolated from all the *R. solanacearum* isolates on quantification was found to be in the range of 300-400 ng μL^{-1}.

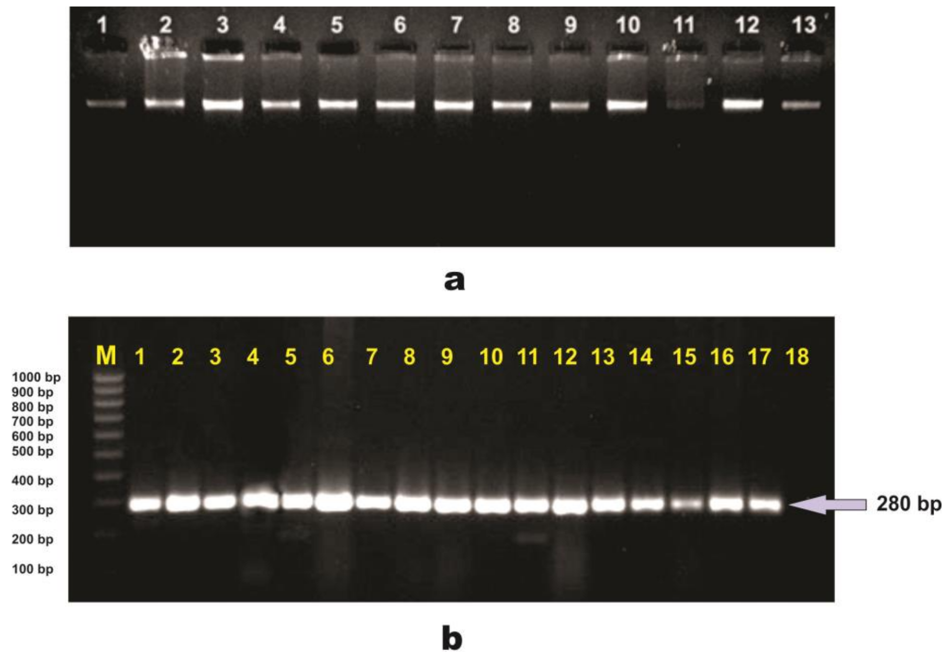


Fig. 2.5 (a) Agarose gel showing genomic DNA of *R. solanacearum*; lanes (1-13) depict gDNA from *R. solanacearum* isolates; (b) Polymerase chain reaction amplification of 280-bp fragment (Rs759/760 primer set) from *R.solanacearum* isolated from various host plants taken under this study. Lane M, 100-bp marker (100bp-1000bp); lanes (1-17) depict representative *R. solanacearum* isolates, lane 18: negative control (water).

2.3.4. Phylotype-specific-multiplex PCR amplification

Phylotype specific-multiplex PCR amplified 144bp phylotype I specific amplicon in addition to 280 bp species specific fragment indicating that the isolates belonged to Phylotype I (Asian Origin) (Fig. 2.6).

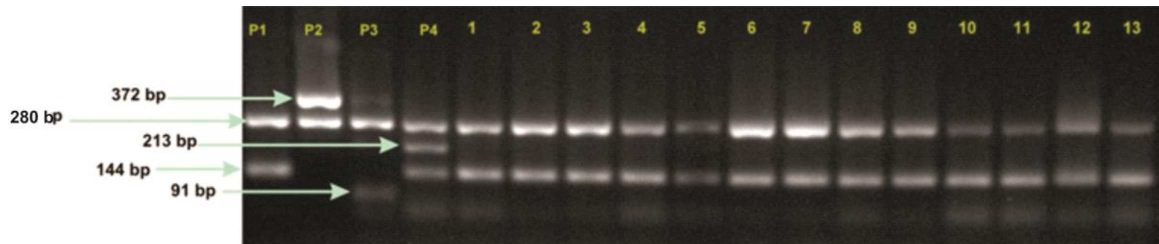


Fig. 2.6 Phylotyping of *R. solanacearum* isolates using specific multiplex PCR. Lanes (P1-P4) represent Phylotype I (GMI1000), II (K60), III (WW386) and IV (WW443) and lanes 1-13 represent *R. solanacearum* isolates (Phylotype I) used in this study. *R. solanacearum* specific amplicon is 282 bp; Phylotype I specific amplicon is 144 bp; Phylotype II specific amplicon is 372 bp; Phylotype III specific amplicon is 91 bp; Phylotype IV specific amplicon is 213 bp.

Fegan and Prior (2005) reported that the species can be divided into 4 phylotypes (I, II, III, IV) based on multiple gene sequence loci which further indicates the geographical origin of the isolates. There is genetic evidence that phylotypes arose from geographical isolation and that populations may have evolved a long time ago (Lavie *et al.*, 2004; Castillo and Greenberg, 2007). According to Cook and Sequeira (1994), the Asiaticum division contains biovars 3, 4, and 5; this proves that all the isolates that belonged to Phylotype I corresponds to Asiaticum Division 1. Although, Sagar *et al.* (2014) has reported that the Indian potato isolates of *R. solanacearum* belong to three out of four phylotypes (I, II, and IV), no other phylotype of *R. solanacearum* infecting solanaceous vegetables was observed. According to Xu *et al.* (2009), the vast majority of isolates analyzed belonged to the Asian phylotype I and biovars 3 and 4.

2.3.5. Pathogenicity and host range studies

R. solanacearum biovar 3 is widely distributed and is known to cause severe losses in tomato, eggplant and other crops.Therefore there was a need to assess the pathogenicity of 50 isolates on eggplant, tomato and chilli seedlings. This was conducted by inoculating 4 mL of bacterial cells (10^8 CFU mL^{-1}) in the root zone of the host plant.

Symptoms like leaf drooping were recorded on daily basis and it was observed that the initial symptoms of wilting in eggplant appeared within 7-10 days but in case of tomato the symptoms appeared within 3-5 days after the pathogen inoculation (Fig. 2.7). Very few isolates showed typical wilting symptoms in the chilli host, but abundant and profuse bacterial streaming was observed in the stem tissues of chilli (Table 2.1).

a

b

Fig. 2.7 Pathogenicity testing of *R. solanacearum* isolates on (a) eggplant host and (b) tomato host in greenhouse exhibiting wilting symptoms. Pathogen inoculum: 8 log CFU mL^{-1}

Table 2.1 Wilt incidences (%) recorded based on the pathogenicity of eggplant, tomato and chilli host range in greenhouse for 30 days period

No.	Isolate	Wilt incidence (%)												
		Eggplant				Tomato *					Chilli			
		7DAI	15DAI	21DAI	28DAI	3DAI	7DAI	15DAI	21DAI	28DAI	7DAI	15 DAI	21 DAI	28 DAI
1	Rs-08-01	0	60	60	60	0	20	40	60	60	0	40	40	40
2	Rs-08-17	0	40	40	40	20	40	60	60	60	0	40	40	40
3	Rs-08-43	0	40	60	60	20	40	40	40	40	40	40	40	40
4	Rs-08-44	0	40	40	40	0	0	20	40	40	0	0	0	0
5	Rs-08-47	40	80	80	80	20	20	40	60	60	0	0	0	0
6	Rs-08-49	60	80	80	80	0	0	0	0	0	0	0	0	0
7	Rs-08-61	40	80	80	80	0	0	0	0	0	0	40	40	60
8	Rs-08-74	0	0	0	20	0	0	0	0	0	0	0	0	0
9	Rs-09-83	100	100	100	100	40	60	60	60	60	0	0	0	0
10	Rs-09-84	60	80	80	80	40	40	40	40	40	0	0	0	0
11	Rs-09-86	80	100	100	100	40	40	60	60	60	0	0	0	0
12	Rs-09-104	80	80	80	80	0	0	40	40	40	0	0	0	0
13	Rs-09-113	80	80	80	80	20	40	60	60	60	0	0	0	0
14	Rs-09-123	20	60	60	60	20	40	60	60	60	0	0	20	40
15	Rs-09-153	20	40	40	40	0	20	40	40	40	0	0	0	0
16	Rs-09-157	20	40	60	60	0	0	20	20	20	0	0	0	0
17	Rs-09-172	40	60	60	60	0	0	40	40	40	0	0	0	0
18	Rs-09-173	60	100	100	100	0	0	0	0	0	0	0	0	0
19	Rs-09-176	20	20	40	40	20	20	40	40	40	0	0	0	0
20	Rs-09-189	0	0	40	40	0	0	0	0	0	0	0	0	0
21	Rs-09-197	0	0	40	40	0	0	40	40	40	0	0	0	0
22	Rs-09-201	0	60	80	80	0	0	60	60	60	0	0	0	0
23	Rs-10-209	40	60	60	60	0	40	60	60	60	0	0	0	0
24	Rs-10-216	0	0	0	0	0	0	0	0	0	0	0	0	0
25	Rs-10-250	40	80	80	80	20	40	40	60	60	0	0	0	0
26	Rs-10-262	40	80	80	80	0	0	40	40	40	0	0	0	0

No.	Isolate	Wilt incidence (%)												
		Eggplant				Tomato*					Chilli			
		7DAI	15DAI	21DAI	28DAI	3DAI	7DAI	15DAI	21DAI	28DAI	7DAI	15 DAI	21 DAI	28 DAI
28	Rs-10-268	0	40	60	60	40	40	40	60	60	0	0	0	0
29	Rs-10-273	40	80	80	80	0	0	60	60	60	0	0	0	0
30	Rs-10-277	0	20	30	40	20	20	60	60	60	0	20	40	40
31	Rs-10-278	40	80	100	100	40	40	40	40	40	40	60	80	80
32	Rs-10-280	0	60	60	80	20	20	60	60	60	0	0	0	0
33	Rs-10-286	0	40	40	40	0	20	40	40	40	20	60	60	60
34	Rs-10-295	40	80	80	80	60	60	80	80	80	0	0	0	0
35	Rs-10-298	30	40	40	40	20	40	40	40	40	0	0	0	0
36	Rs-10-315	40	80	80	80	40	40	60	60	60	40	40	60	60
37	Rs-10-319	40	60	80	80	40	40	40	40	40	0	0	0	0
38	Rs-10-322	0	60	80	80	40	40	60	60	60	0	0	0	0
39	Rs-10-325	0	40	60	60	60	60	100	100	100	0	0	0	0
40	Rs-10-332	0	20	40	40	20	40	60	60	60	0	0	0	0
41	Rs-10-337	20	80	100	100	40	40	60	60	60	0	0	0	0
42	Rs-11-340	0	20	60	60	40	60	60	60	80	0	0	0	0
43	Rs-11-341	20	20	20	20	0	20	40	60	60	0	0	0	0
44	Rs-11-344	0	30	40	60	0	40	60	60	80	0	0	0	0
45	Rs-11-357	0	0	20	20	0	40	60	60	60	0	0	0	0
46	Rs-11-359	20	60	60	80	0	40	60	80	100	0	40	40	40
47	Rs-11-363	40	40	60	60	40	60	100	100	100	0	20	40	40
48	Rs-11-364	20	20	20	20	0	40	100	100	100	0	0	0	0
49	Rs-11-365	20	40	40	40	0	60	80	80	80	0	0	0	0
50	Rs-11-376	60	80	80	80	40	60	60	60	60	0	0	0	0

Initial inoculum: 8 log CFU mL^{-1}; DAI- days after inoculation; 10 plants were inoculated per isolate

*Tomato wilt started early within 3-5 days.

Results indicated that out of the 50 isolates taken under the study, 49 isolates were pathogenic on eggplant and 44 isolates were pathogenic on tomato (Table 2.2). It was observed that Rs-08-83, Rs-08-86 and Rs-09-173 wilted 100% eggplants within 7, 15 and 15 days after inoculation respectively. In case of tomato pathogenicity, 5 isolates viz. Rs-08-49, Rs-08-61, Rs-08-74, Rs-09-173 and Rs-09-189 failed to cause infection in the host even after 30 days of inoculation. However, 3 isolates (Rs-10-325, Rs-11-363 and Rs-11-364) wilted 100% tomato plants within 15 days of inoculation (Table 2.1). Four isolates viz. Rs-08-49, Rs-08-74, Rs-09-173 and Rs-09-189 caused wilting only in eggplant (Table 2.2). Only 11 isolates viz. Rs-08-01, Rs-08-17, Rs-08-43, Rs-08-61, Rs-10-266, Rs-10-277, Rs-10-278, Rs-10-295, Rs-10-315, Rs-09-123 and Rs-11-363 successfully caused wilting in chilli host (Table 2.2).

Table 2.2 Host range studies on 3 hosts and their classification into pathogenic groups

No.	**Isolate**	**Host**	**Origin**	**Pathogenicity testing**			**Pathogenic Group**
				Eggplant	Tomato	Chilli	I/II/III/IV
1	Rs-08-01	Eggplant	Goa	+	+	+	I
2	Rs-08-17	Eggplant	Goa	+	+	+	I
3	Rs-08-43	Eggplant	Goa	+	+	+	I
4	Rs-08-44	Eggplant	Goa	+	+	-	II
5	Rs-08-47	Eggplant	Goa	+	+	-	II
6	Rs-08-49	Eggplant	Goa	+	-	-	IV
7	Rs-08-61	Eggplant	Goa	+	-	+	III
8	Rs-08-74	Eggplant	Goa	+	-	-	IV
9	Rs-09-84	Eggplant	Goa	+	+	-	II
10	Rs-09-86	Eggplant	Goa	+	+	-	II
11	Rs-09-104	Eggplant	Goa	+	+	-	II
12	Rs-09-153	Eggplant	Goa	+	+	-	II
13	Rs-09-157	Eggplant	Goa	+	+	-	II
14	Rs-09-172	Eggplant	Goa	+	+	-	II
15	Rs-09-173	Eggplant	Goa	+	-	-	IV
16	Rs-09-176	Eggplant	Goa	+	+	-	II
17	Rs-10-262	Eggplant	Goa	+	+	-	II
18	Rs-10-266	Eggplant	Goa	+	+	+	I
19	Rs-10-268	Eggplant	Goa	+	+	-	II
20	Rs-10-273	Eggplant	Goa	+	+	-	II

No.	Isolate	Host	Origin	Pathogenicity testing			Pathogenic Group
				Eggplant	Tomato	Chilli	I/II/III/IV
21	Rs-10-277	Eggplant	Goa	+	+	+	I
22	Rs-10-278	Eggplant	Goa	+	+	+	I
23	Rs-10-286	Eggplant	Goa	+	+	-	II
24	Rs-10-295	Eggplant	Goa	+	+	+	I
25	Rs-10-298	Eggplant	Goa	+	+	-	II
26	Rs-10-315	Eggplant	Goa	+	+	+	I
27	Rs-10-319	Eggplant	Goa	+	+	-	II
28	Rs-10-322	Eggplant	Goa	+	+	-	II
29	Rs-10-325	Eggplant	Goa	+	+	-	II
30	Rs-10-337	Eggplant	Goa	+	+	-	II
31	Rs-10-280	Chilli	Goa	+	+	-	II
32	Rs-10-332	Chilli	Goa	+	+	-	II
33	Rs-09-83	Tomato	Goa	+	+	-	II
34	Rs-09-197	Chilli	Karnataka	+	+	-	II
35	Rs-09-201	Chilli	Karnataka	+	+	-	II
36	Rs-09-189	Potato	Karnataka	+	-	-	IV
37	Rs-09-123	Eggplant	Kerala	+	+	+	I
38	Rs-09-113	Chilli	Kerala	+	+	-	II
39	Rs-10-209	Tomato	Kerala	+	+	-	II
40	Rs-10-216	Ginger	Kerala	-	-	-	V
41	Rs-11-376	Eggplant	Orissa	+	+	-	II
42	Rs-11-357	Eggplant	Maharashtra	+	+	-	II
43	Rs-11-359	Eggplant	Maharashtra	+	+	-	II
44	Rs-11-364	Eggplant	Maharashtra	+	+	-	II
45	Rs-11-363	Eggplant	Maharashtra	+	+	+	I
46	Rs-11-365	Eggplant	Maharashtra	+	+	-	II
47	Rs-11-340	Eggplant	Maharashtra	+	+	-	II
48	Rs-11-341	Eggplant	Maharashtra	+	+	-	II
49	Rs-11-344	Eggplant	Maharashtra	+	+	-	II
50	Rs-10-250	Eggplant	Andaman	+	+	-	II

+ Pathogenic, - non-pathogenic; Group I - Pathogenic on all the 3 hosts, Group II- Pathogenic on eggplant and tomato host; Group III - Pathogenic on eggplant and chilli host; Group IV- Pathogenic on eggplant host only; Group V – non-pathogenic on all the hosts.

Early classification of *R. solanacearum* divided the species into five major races based on the host range of the isolates, and these ‘races’ mostly corresponded to pathovars (Denny, 2006). Kumar and Sarma (2004) also reported that *R. solanacearum* isolates from ginger (Race4/Biovar3) are highly pathogenic on ginger as well as on solanaceous vegetables. However, in the present study the ginger isolate Rs-10-216 (Race 4/Biovar 3) did not

cause wilt in any of the host tested (Table 2.1, 2.2). Similar kind of observation was recorded by Mondal *et al.* (2011) regarding ginger isolate being non-pathogenic on solanaceous vegetables. The incidence of biovar 4 (Kumar and Sarma, 2004) is less frequent in India, which further confirms that biovar 3 is more versatile in its adaptation to varying environmental conditions

After 15 days of inoculation, 5, 6 and 39 isolates didn't cause wilt on eggplant, tomato and chilli respectively (Table 2.3). Eggplant cultivar and tomato variety used in this experiment were highly susceptible to bacterial wilt which might be a reason for higher disease incidence. However, chilli variety used is moderately resistant to bacterial wilt as it is evident from the results of the study by Ramesh *et al.* (2014b). However, few isolates which did not cause wilting after 15 days showed wilting after 17 days of inoculation on eggplant and tomato. In case of chilli, the isolates which did not cause typical wilt after 15 days of inoculation remained chlorotic till the end of the study period.

Table 2.3 Number of isolates pathogenic on eggplant, tomato and chilli after 15 days of inoculation

Host Name	**Wilt Percentage (%) after 15 DAI**				
	0%	<25 %	26-50 %	51-75 %	76-100 %
Eggplant	5	6	12	9	18
Tomato	6	2	18	19	5
Chilli	39*	3	6	2	0

Initial inoculum: 8 log CFU mL; *Out of the 39 isolates only 11 isolates showed typical wilting symptoms in chilli plants

Based on host range studies, it could be concluded that the isolates were pathogenic on solanaceous plants such as eggplant, tomato and chilli which corresponded to Race 1. Bhattacharya *et al.* (2003) reported the prevalence of Race 1 and biovar 3 infecting solanaceous crops from West Bengal. Shekhawat *et al.* (1978) reported that isolates from

plain area belonged to Race 1. He also reported that biovar 3 and 4 were encountered only among eastern parts of India but our findings revealed that they all belonged to biovar 3 Race 1 from western parts of India. Moreover, *R. solanacearum* have also been categorised into Race 1 if they successfully infected tomato and other solanaceaous crops (OEPP/EPPO, 2004).

Earlier many researchers have classified *R. solanacaerum* isolates based on their ability to cause wilt in different hosts (Buddenhagen *et al.*, 1962; Horita and Tsuchiya, 2001). Therefore the isolates were classified into 4 pathogenic groups. Group I included isolates that were pathogenic on all the 3 hosts (10 isolates); group II comprised of isolates that were pathogenic on eggplant and tomato host only (44 isolates); group III included only a single isolate that was pathogenic on eggplant and chilli host and group IV comprised of isolates which were only pathogenic on eggplant host (4 isolates) and group V which are non-pathogenic on all the hosts (Table 2.2). Jaunet and Wang (1999) determined the pathogenic variation among the isolates present in tomato fields and hypothesised that the variability that was observed in *R. solanacearum* population can also be related to its soilborne nature. From literature, it's a known fact that the most virulent and aggressive phylotype I isolates belonging to biovar 3, 4, and 5 have only been reported in Asia (Truong *et al.*, 2008). Jaunet and Wang (1999) recorded that all the isolates produced wilting symptoms in tomato plants after 15 days post inoculation and were considered pathogenic whereas, in this host range study the wilting was recorded within a very short period of time in tomato (3-5 days post inoculation). Ji *et al.* (2007) reported that biovar type and aggressiveness of *R. solanacearum* may be linked. However, this study does not provide any evidence to link aggressiveness to biovar as all the isolates were biovar 3

(except one isolate) and a wide variation in their pathogenicity was observed on different hosts. Horita and Tsuchiya (2001) stated that biovar 3 isolates differ in their pathogenicity and my findings are in the same line with their report.

Results are presented in determining the potential of the pathogen to cause wilt after 15 days of inoculation (Table 2.3). It indicated that on eggplant, 18 isolates wilted 76-100% plants, 9 isolates wilted 51-75% plants, 12 isolates wilted 26-50% plants and 6 isolates wilted less than 25% plants after 15 days of inoculation. Likewise, on tomato, only 5 isolates wilted 76-100% plants, 19 isolates wilted 51-75% plants, 18 isolates wilted 26-50% plants and only 2 isolates wilted less than 25% plants after 15 days of inoculation. On chilli, none of the isolates wilted more than 75% of the inoculated plants, 2 isolates wilted 51-75% plants, 6 isolates wilted 26-50% plants and 3 isolates wilted less than 25% plants. Overall it was observed that 36% of isolates wilted 76-100% of eggplant and only 10% of isolates wilted the same number of tomato plants. *R. solanacearum* biovar 3 is widely distributed and is known to cause severe losses in tomato, eggplant and other crops (Elphinstone, 2005; Raymundo *et al.*, 2005).

2.3.6. Biochemical tests

On the basis of biochemical tests performed it was found that the isolates produced thin slimy strands when mixed with 3% KOH solution (Fig. 2.8 a). Also the isolates where found to be catalase positive as they produced gas bubbles when mixed with a drop of H_2O_2 on glass slide (Fig. 2.8 b). None of the isolates hydrolyzed gelatin and hence regarded gelatinase negative. Isolates showed positive activity for oxidase test. All the isolates reduced nitrate to nitrite. The isolates grew at 37°C but failed to survive at 41°C.

The isolates produced brown pigmentation on King's B medium and were non-fluorescent in nature.

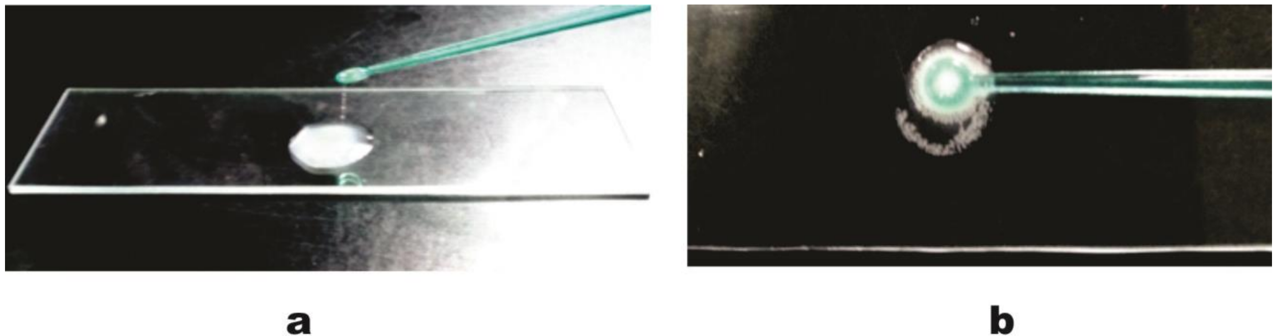


Fig. 2.8 Biochemical tests: (a) Potassium hydroxide test showing slimy threads and (b) Catalase test exhibiting air bubbles.

Overall, the observations recorded for biochemical tests proved that the pathogen is catalase and oxidase positive and a nitrate reducer by nature. These findings are in conformity with the findings of Kelman (1954) and Hayward (1994).

With this background information, further an attempt was made to genetically characterize *R. solanacearum* isolates by ARDRA, PCR-RFLP, Rep-PCR and sequencing studies.

2.3.7. Genetic characterization of *R. solanacearum* isolates

2.3.7.1. ARDRA and PCR-RFLP of *R. solanacearum* genes

16S rRNA, *fliC* gene and *lpxC* gene fragments from *R. solanacearum* isolates (Table 2 C) from this study belonging to Phylotype I and other *R. solanacearum* reference isolates (Phylotype I, II, III and IV) were amplified (Fig. 2.9) and later digested with different restriction enzymes.

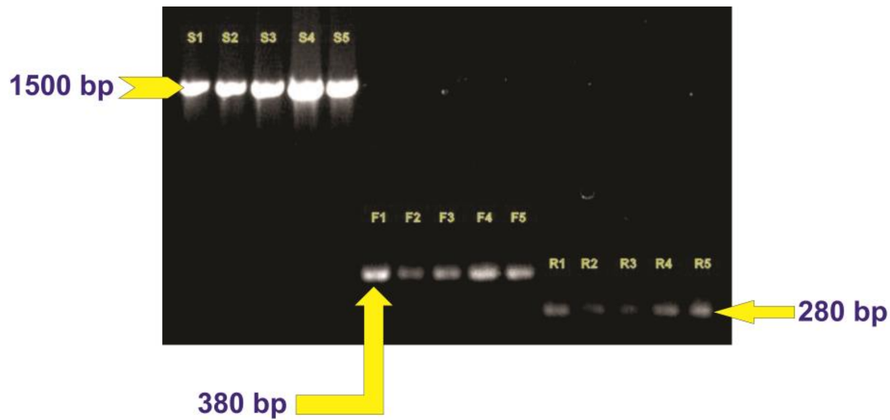


Fig. 2.9 PCR amplification of 16S rRNA gene (lanes S1-S5), *fliC* gene (F1-F5) and 280 bp gene (R1-R5) resolved by 1.5% agarose gel electrophoresis.

Amplified ribosomal DNA restriction analysis (ARDRA)

It was observed that the restriction profiles obtained from the Phylotype I isolates with different restriction endonucleases produced uniform and identical patterns in all the 3 genes used with various enzyme combinations. However, polymorphic bands were identified when the template DNA from Phylotype II, III and IV were employed. 16S rRNA fragment restricted with *Alu* I did not differentiate the *R. solanacearum* isolates (Fig. 2.10 a, b), whereas, *Msp* I differentiated phylotype II from other phylotypes with a single extra polymorpic band of size 500bp approximately (Fig. 2.10 c, d).

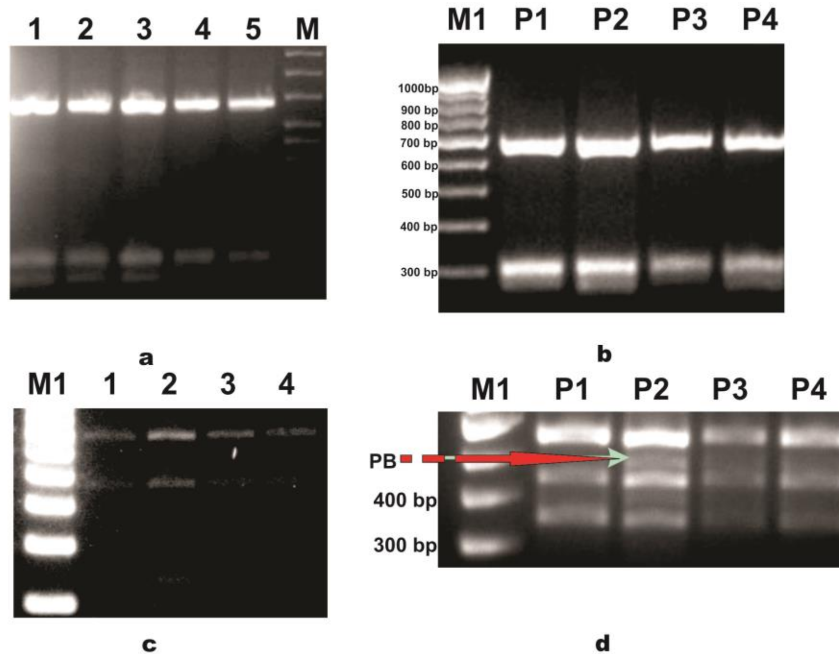


Fig. 2.10 ARDRA profile of *R. solanacearum* isolates digested with *Alu* I (a, b) and *Msp* I (c, d). Lanes (P1-P4) represent Phylotypes I, II, III and IV; lanes (1-5) depict *R. solanacearum* strains. M represents 50 bp step ladder (Sigma) (DNA size marker). M1 represents 100bp (Genei) (DNA size marker). PB refers to the polymorphic band.

PCR-RFLP of the *fliC* gene and 280 bp gene fragment

When *fliC* gene was restricted with *Alu* I, only phylotype II showed different restriction profile with an additional fragment of 100bp (Fig. 2.11).

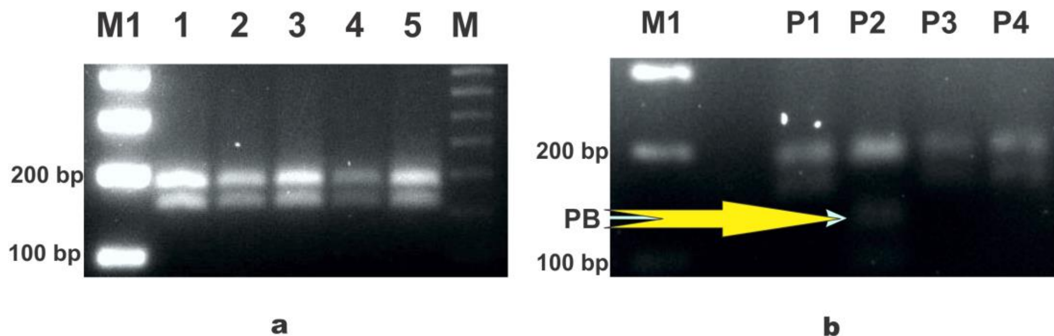


Fig. 2.11 Restriction fragment length polymorphisms of polymerase chain reaction-amplified *fliC* gene fragments of of *R. solanacearum* isolates. Restriction patterns were generated with *Alu* I (a, b). Lanes (P1-P4) represent Phylotypes I, II, II and IV; lanes (1-5) depict *R. solanacearum* isolates. PB refers to Polymorphic band. M1 represents 100bp (Genei) (DNA size marker), M represents 50bp step ladder (Sigma) (DNA size marker).

In case of PCR-RFLP of 280 bp gene with *Nla* III, 3 different fingerprint patterns were produced. Phylotype I and phylotype IV produced single identical restriction profile, whereas phylotype II and Phylotype III produced different profiles (Fig. 2.12).

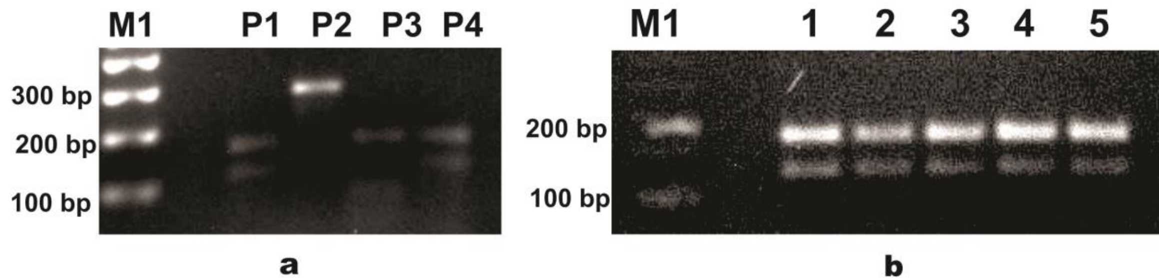


Fig. 2.12 Restriction fragment length polymorphisms of polymerase chain reaction-amplified 280 bp fragments of different strains of *R. solanacearum*. Restriction patterns were generated with *Nla* III enzyme (a, b). Lanes (P1-P4) represent Phylotypes I, II, II and IV; lanes (1-5) depict *R. solanacearum* isolates. M represents 100bp (Genei) (DNA size marker).

Molecular techniques viz. ARDRA and PCR-RFLP fingerprinting were used to classify the isolates to assess the diversity and genetic relationships among Phylotype I biovar 3 *R. solanacearum* isolates. However, no polymorphism was observed in all the three genes used with various enzyme combinations. The results are in agreement with the reports of Xue *et al.* (2010) wherein all isolates under study also displayed a distinct *fliC*-RFLP pattern. Most strains assigned to phylotype I by multiplex PCR displayed identical *fliC*-RFLP patterns, the results of this study therefore supported the findings of Castillo and Greenberg (2007) that housekeeping genes such as *fliC* have been subjected to purifying selection.

However, Thammakijjawat *et al.* (2004) used PCR-RFLP with Rs759/760 primers and restricted with *Hae* III and *Msp* I and it differentiated the biovar 2 Race 3 *R. solanacearum* from biovars 3, 4 and N2. The results of this study are not in agreement with the RFLP analysis of a specific 280-bp fragment in *R. solanacearum* conducted by

Villa *et al.* (2003) who reported that the isolates present in Asia (phylotype I) showed distinction between different biovars generating 3 different RFLP patterns. This could have happened because all the isolates under this study belonged to phylotype I biovar 3 and hence no distinct variation. However, Polymorphism was observed in the restriction profile when different phylotype isolates were compared in these studies.

2.3.7.2. Rep-PCR analysis

Rep-PCR analysis was therefore performed on 50 *R. solanacearum* isolates representing 6 states from India. PCR data was converted into binary data and analyzed using NTSYSpc 2.02i software. Dendrogram was generated and clustering was done by the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NYSYS-pc.

BOX PCR analysis

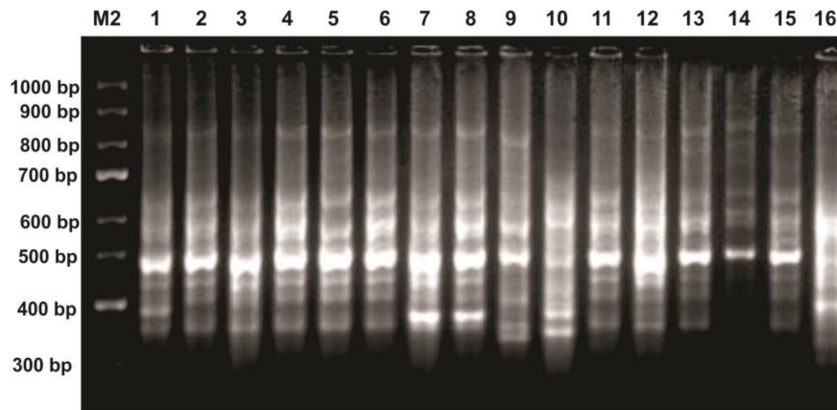
When BOX PCR fingerprints (Fig. 2.13 A) were analyzed, isolates from Goa showed as many as 8 distinct fingerprint patterns, Maharashtra isolates showed 5 patterns, Karnataka isolates showed 3 patterns, Kerala isolates showed 4 patterns, Orissa and Andaman isolate showed a single unique pattern each (Table 2.4).

Table 2.4 Grouping of *R. solanacearum* based on BOX and ERIC PCR fingerprints and clusters

State	BOX PCR			ERIC PCR	
	Total no. of isolates	No. of distinct patterns[a]	No. of Clusters[b]	No. of distinct patterns[a]	No. of Clusters[b]
Goa	33	8	6	6	6
Maharashtra	8	5	4	5	5
Karnataka	3	3	3	3	3
Kerala	4	4	4	3	3
Andaman	1	1	1	1	1
Orissa	1	1	1	1	1

[a]Total number of fingerprint patterns pertaining to individual state

[b]Total number of isolates grouped in a cluster at 75% similarity coefficient



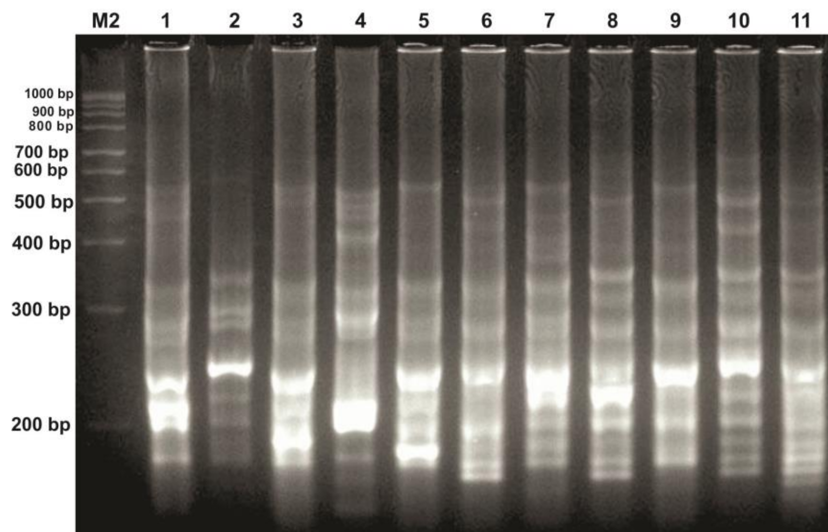


Fig. 2.13 Representative rep-PCR patterns generated by amplifying genomic DNA of representative strains of *R. solanacearum* with (A) BOXAIR, lanes (1-16): representative *R. solanacearum* isolates and (B) ERIC primers, lanes (1-11): representative *R. solanacearum* isolates. Lane M2, DNA size marker (1 Kb ladder).

Isolates from Goa exclusively produced 7 distinct fingerprint patterns (P1, P6, P7, P8, P9, P13 and P16). Nineteen isolates from Goa showed a distinct fingerprint pattern P2. Interestingly, a single isolate each from Goa, Kerala and Maharashtra exhibited identical fingerprint pattern P11. One potato isolate (Rs-09-189) from Karnataka and one eggplant

isolate (Rs-09-123) from Kerala shared the same fingerprint pattern P3. Identical fingerprint pattern (P14) was produced by an isolate from Maharashtra, Orissa and Karnataka each, along with 2 isolates from Goa. Rs-09-113 (biovar 6) isolate produced identical fingerprint pattern with biovar 3 isolates from Goa (Rs-10-325) and Maharashtra (Rs-11-364). Rs-09-83 isolate from Goa isolated from tomato host also formed a unique fingerprint P16 (Table 2.5; Fig. 2.14).

Table 2.5 BOX fingerprint patterns (P) produced by 50 *R. solanacearum* isolates

Patterns	Isolate	Crop	State	Total no. of isolates
P1	Rs-10-266	Eggplant	Goa	4
	Rs-08-44	Eggplant		
	Rs-09-153	Eggplant		
	Rs-10-262	Eggplant		
P2	Rs-08-01	Eggplant	Goa	19
	Rs-08-17	Eggplant		
	Rs-08-43	Eggplant		
	Rs-08-47	Eggplant		
	Rs-08-49	Eggplant		
	Rs-08-74	Eggplant		
	Rs-09-84	Eggplant		
	Rs-09-86	Eggplant		
	Rs-09-104	Eggplant		
	Rs-09-157	Eggplant		
	Rs-09-173	Eggplant		
	Rs-10-268	Eggplant		
	Rs-10-273	Eggplant		
	Rs-10-295	Eggplant		
	Rs-10-298	Eggplant		
	Rs-10-315	Eggplant		
	Rs-10-319	Eggplant		
	Rs-10-322	Eggplant		
	Rs-10-332	Chilli		
	Rs-10-209	Tomato	Kerala	1
	Rs-09-197	Chilli	Karnataka	1
P3	Rs-09-189	Potato	Karnataka	1
	Rs-09-123	Eggplant	Kerala	1
P4	Rs-11-344	Eggplant	Maharashtra	1
P5	Rs-11-340	Eggplant	Maharashtra	3
	Rs-11-341	Eggplant		
	Rs-11-357	Eggplant		
P6	Rs-10-337	Eggplant	Goa	1
P7	Rs-10-286	Eggplant	Goa	1

Patterns	Isolate	Crop	State	Total no. of isolates
P8	Rs-08-61	Eggplant	Goa	1
P9	Rs-10-280	Chilli	Goa	1
P10	Rs-10-250	Eggplant	Andaman	1
P11	Rs-10-325	Eggplant	Goa	1
	Rs-09-113	Chilli	Kerala	1
	Rs-11-364	Eggplant	Maharashtra	1
P12	Rs-11-363	Eggplant	Maharashtra	2
	Rs-11-365	Eggplant	Maharashtra	
P13	Rs-09-172	Eggplant	Goa	2
	Rs-09-176	Eggplant		
P14	Rs-10-278	Eggplant	Goa	2
	Rs-10-277	Eggplant		
	Rs-11-359	Eggplant	Maharashtra	1
	Rs-11-376	Eggplant	Orissa	1
	Rs-09-201	Chilli	Karnataka	1
P15	Rs-10-216	Ginger	Kerala	1
P16	Rs-09-83	Tomato	Goa	1

At 75% similarity coefficient it was observed that BOX PCR cluster analysis produced 10 clusters (Fig. 2.14) Maximum number of isolates (50%) was present in Cluster I that comprised of isolates collectively from Goa (n=23), Karnataka (n=1) and Kerala (n=1) (Table 2.6). Four clusters (C-II, C-III, C-VII and C-X) comprised 5 isolates exclusively from Goa forming independent clusters. Cluster I comprised of isolates which were isolated from eggplant and one isolate each from Karnataka (chilli) and Kerala (tomato). Cluster II, VII, VIII and X comprised of a single isolate each. Cluster IV belonged to isolates from Maharashtra only. Cluster V comprised of mixed group of isolates that belonged to Goa, Maharashtra, Karnataka, Orissa and Andaman. Ginger isolate from Kerala formed a separate cluster (C-VIII). Biovar 6 isolate Rs-09-113 belonged to cluster IX along with three more isolates from Goa and Maharashtra. The two main groups were distinguished at a mean level of similarity of 30%.

Table 2.6 Clustering of BOX PCR produced by 50 isolates of *R. solanacearum* and the corresponding pathogenic group

Cluster	Isolate	Host	State	Number of isolates	Pathogenic group
C I	Rs-10-266	Eggplant	Goa	23	
	Rs-08-44	Eggplant			
	Rs-09-153	Eggplant			
	Rs-10-262	Eggplant			I, II and IV
	Rs-08-01	Eggplant			
	Rs-08-17	Eggplant			
	Rs-08-43	Eggplant			
	Rs-08-47	Eggplant			
	Rs-08-49	Eggplant			
	Rs-08-74	Eggplant			
	Rs-09-84	Eggplant			
	Rs-09-86	Eggplant			
	Rs-09-104	Eggplant			
	Rs-09-157	Eggplant			
	Rs-09-173	Eggplant			
	Rs-10-268	Eggplant			
	Rs-10-273	Eggplant			
	Rs-10-295	Eggplant			
	Rs-10-298	Eggplant			
	Rs-10-315	Eggplant			
	Rs-10-319	Eggplant			
	Rs-10-322	Eggplant			
	Rs-10-332	Chilli			
	Rs-10-209	Tomato	Kerala	1	
	Rs-09-197	Chilli	Karnataka	1	
C II	Rs-08-61	Eggplant	Goa	1	III
C III	Rs-09-172	Eggplant	Goa	2	II
	Rs-09-176	Eggplant			
C IV	Rs-11-344	Eggplant	Maharashtra	4	II
	Rs-11-340	Eggplant			
	Rs-11-341	Eggplant			
	Rs-11-357	Eggplant			
C V	Rs-10-278	Eggplant	Goa	3	I and II
	Rs-10-277	Eggplant			
	Rs-09-83	Tomato			
	Rs-11-359	Eggplant	Maharashtra	1	
	Rs-11-376	Eggplant	Orissa	1	
	Rs-09-201	Chilli	Karnataka	1	
	Rs-10-250	Eggplant	Andaman	1	
C VI	Rs-11-363	Eggplant	Maharashtra	2	I, II and IV
	Rs-11-365	Eggplant			
C VI	Rs-09-189	Potato	Karnataka	1	
	Rs-09-123	Eggplant	Kerala	1	
C VII	Rs-10-337	Eggplant	Goa	1	II
C VIII	Rs-10-216	Ginger	Kerala	1	V
C IX	Rs-10-280	Chilli	Goa	1	

Cluster	Isolate	Host	State	Number of isolates	Pathogenic group
	Rs-10-325	Eggplant			II
	Rs-09-113	Chilli	Kerala	1	
	Rs-11-364	Eggplant	Maharashtra	1	
C X	Rs-10-286	Eggplant	Goa	1	II

C- Cluster; Pathogenic groups were based on the ability of isolates to cause wilt in different hosts (Refer Table 2.2)

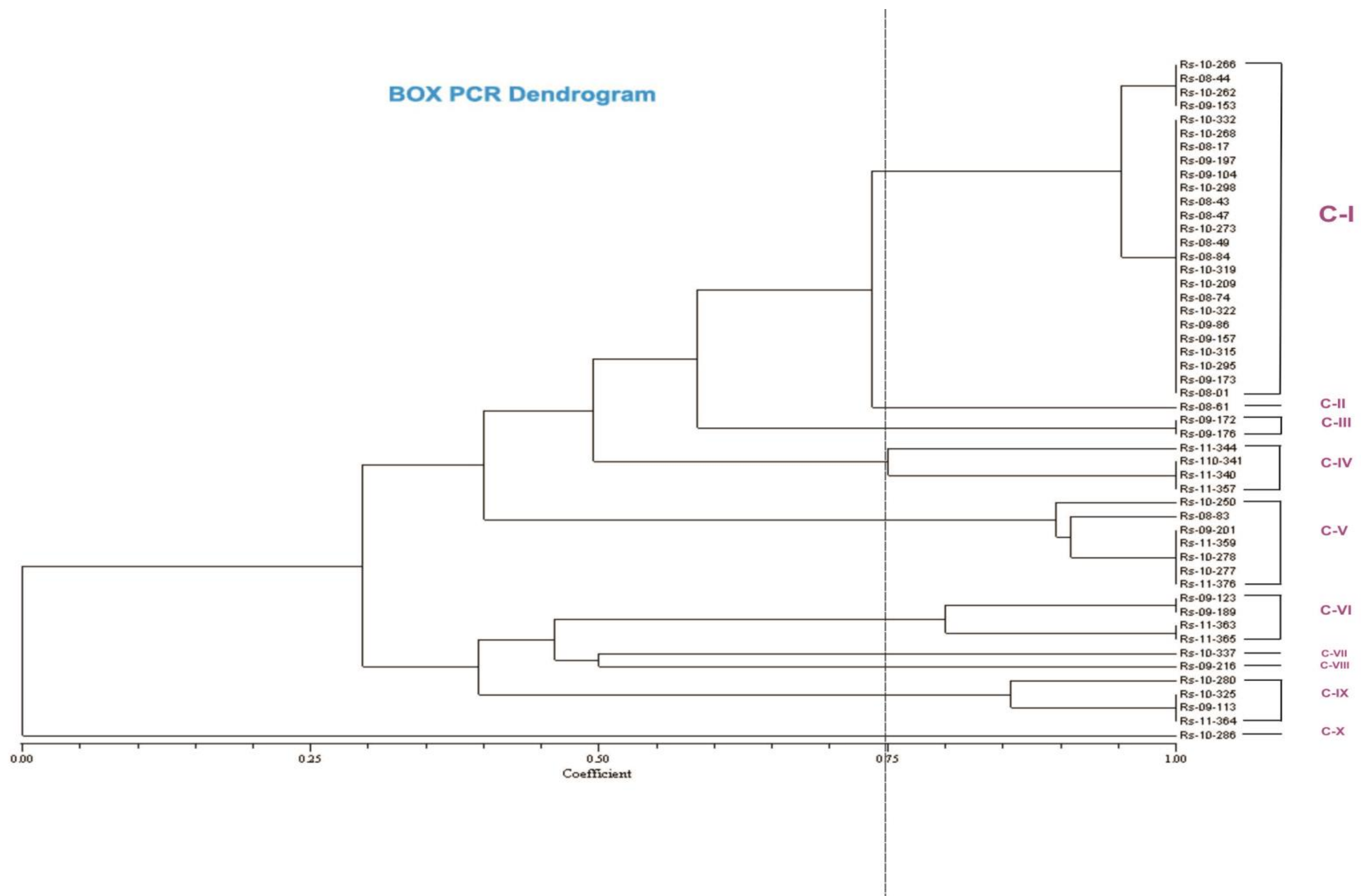


Fig. 2.14 Dendrogram showing the relationship among 50 *R. solanacearum* isolates from India based on rep-PCR using BOXAIR primer. Similarities and clustering (NTSYSpc software v 2.02i, Applied Biostatistics Inc. USA) were calculated by using the Dice coefficient and UPGMA showing phylogenetic relationships.

ERIC PCR analysis

When ERIC PCR fingerprints (Fig 2.13 B) were analyzed, isolates from Goa showed as many as 6 distinct fingerprint patterns, Maharashtra isolates showed 5 patterns, Karnataka and Kerala isolates showed 3 patterns each, Orissa and Andaman isolate showed a single unique pattern each (Table 2.4)

Table 2.7 ERIC fingerprint patterns (P) produced by 50 isolates of *R. solanacearum*

Pattern	Isolate	Crop	State	Total no. of isolates
P1	Rs-09-153	Eggplant	Goa	12
	Rs-08-01	Eggplant		
	Rs-08-17	Eggplant		
	Rs-08-43	Eggplant		
	Rs-08-47	Eggplant		
	Rs-08-49	Eggplant		
	Rs-08-74	Eggplant		
	Rs-09-84	Eggplant		
	Rs-09-157	Eggplant		
	Rs-10-273	Eggplant		
	Rs-09-176	Eggplant		
	Rs-10-337	Eggplant		
	Rs-11-364	Eggplant	Maharashtra	2
	Rs-11-365	Eggplant		
P2	Rs-09-173	Eggplant	Goa	8
	Rs-09-172	Eggplant		
	Rs-10-286	Eggplant		
	Rs-09-83	Eggplant		
	Rs-10-295	Eggplant		
	Rs-10-266	Eggplant		
	Rs-10-268	Eggplant		
	Rs-10-262	Eggplant		
P3	Rs-10-277	Eggplant	Goa	5
	Rs-10-325	Eggplant		
	Rs-10-332	Eggplant		
	Rs-08-44	Eggplant		
	Rs-10-315	Eggplant		
	Rs-11-344	Eggplant	Maharashtra	1
P4	Rs-10-278	Eggplant	Goa	4
	Rs-10-298	Eggplant		
	Rs-10-319	Eggplant		
	Rs-10-322	Eggplant		
P5	Rs-11-340	Eggplant	Maharashtra	3
	Rs-11-341	Eggplant		
	Rs-11-359	Eggplant		

Pattern	Isolate	Crop	State	Total no. of isolates
P6	Rs-09-113	Chilli	Kerala	2
	Rs-09-123	Eggplant		
	Rs-09-197	Chilli	Karnataka	1
P7	Rs-09-86	Eggplant	Goa	2
	Rs-10-280	chilli		
	Rs-09-201	Chilli	Karnataka	1
P8	Rs-08-61	Eggplant	Goa	2
	Rs-09-104	Eggplant		
	Rs-10-209	Tomato	Kerala	1
P9	Rs-11-357	Eggplant	Maharashtra	1
P10	Rs-10-216	Ginger	Kerala	1
P11	Rs-11-363	Eggplant	Maharashtra	1
P12	Rs-11-376	Eggplant	Orissa	1
P13	Rs-10-250	Eggplant	Andaman	1
P14	Rs-09-189	Potato	Karnataka	1

Isolates from Goa exclusively produced 2 distinct fingerprint patterns (P2 and P4). Twelve isolates from Goa showed a distinct fingerprint pattern P1, followed by 8 isolates that exhibited P2 pattern. Maharashtra isolates produced 3 patterns exclusively (P5, P9 and P11). Pattern P6 was produced by isolates from Kerala and Karnataka. *R. solanacearum* isolated from ginger (Rs-10-216), Orissa (Rs-11-376), Maharashtra (Rs-11-363), Karnataka (Rs-09-189) and Andaman (Rs-10-250) showed a very distinct fingerprint pattern. Rs-08-113 (biovar 6) isolate shared identical fingerprint pattern with biovar 3 isolates from Karnataka (n=2) (Table 2.7).

ERIC cluster analysis resulted in identification of 14 clusters at 75% similarity coefficient. Out of these, 2 clusters (C- II and C- IV) belong to isolates from Goa (Table 2.8; Fig 2.15). Cluster I had maximum number of isolates from Goa (n=12) in addition to 2 isolates from Maharashtra. Cluster V comprised of isolates from Maharashtra only. Cluster III comprised of isolates only from Goa except one isolate (Rs-11-344) that belonged to Maharashtra. Cluster VII consisted of eggplant and chilli isolates from Goa (n=2) and 1 isolate (Rs-09-201) from Karnataka. Individual clusters were formed by

isolates from Maharashtra (C-IX, C-XI), Kerala (C-X), Orissa (X-II), Andaman (X-III) and Karnataka (C-XIV) each. The two main groups were distinguished at a mean level of similarity of 35%.

Table 2.8 Clustering of ERIC fingerprint patterns produced by 50 isolates of *R. solanacearum* and their pathogenic group

Cluster	Isolate	Crop	State	Total number of isolates	Pathogenic group
C I	Rs-09-153	Eggplant	Goa	12	
	Rs-08-01	Eggplant			
	Rs-08-17	Eggplant			
	Rs-08-43	Eggplant			
	Rs-08-47	Eggplant			
	Rs-08-49	Eggplant			I, II and IV
	Rs-08-74	Eggplant			
	Rs-09-84	Eggplant			
	Rs-09-157	Eggplant			
	Rs-10-273	Eggplant			
	Rs-09-176	Eggplant			
	Rs-10-337	Eggplant			
	Rs-11-364	Eggplant	Maharashtra	2	
	Rs-11-365	Eggplant			
C II	Rs-09-173	Eggplant	Goa	8	
	Rs-09-172	Eggplant			
	Rs-10-286	Eggplant			I, II and IV
	Rs-09-83	Tomato			
	Rs-10-295	Eggplant			
	Rs-10-266	Eggplant			
	Rs-10-268	Eggplant			
	Rs-10-262	Eggplant			
C III	Rs-10-277	Eggplant	Goa	5	I and II
	Rs-10-325	Eggplant			
	Rs-10-332	Chilli			
	Rs-08-44	Eggplant			
	Rs-10-315	Eggplant			
	Rs-11-344	Eggplant	Maharashtra	1	
C IV	Rs-10-278	Eggplant	Goa	4	I and II
	Rs-10-298	Eggplant			
	Rs-10-319	Eggplant			
	Rs-10-322	Eggplant			
C V	Rs-11-340	Eggplant	Maharashtra	3	II
	Rs-11-341	Eggplant			
	Rs-11-359	Eggplant			
C VI	Rs-09-113	Chilli	Kerala	2	
	Rs-09-123	Eggplant			I and II
	Rs-09-197	Chilli	Karnataka	1	
C VII	Rs-09-86	Eggplant	Goa	2	II

Cluster	Isolate	Crop	State	Total number of isolates	Pathogenic group
C VIII	Rs-10-280	Chilli	Karnataka	1	II and III
	Rs-09-201	Chilli			
	Rs-08-61	Eggplant	Goa	2	
	Rs-09-104	Eggplant			
	Rs-10-209	Tomato	Kerala	1	
C IX	Rs-11-357	Eggplant	Maharashtra	1	II
C X	Rs-10-216	Ginger	Kerala	1	V
C XI	Rs-11-363	Eggplant	Maharashtra	1	I
C XII	Rs-11-376	Eggplant	Orissa	1	II
C XIII	Rs-10-250	Eggplant	Andaman	1	II
C XIV	Rs-09-189	Potato	Karnataka	1	IV

C- Cluster; Pathogenic groups were based on the ability of isolates to cause wilt in different hosts (Refer Table 2.2)

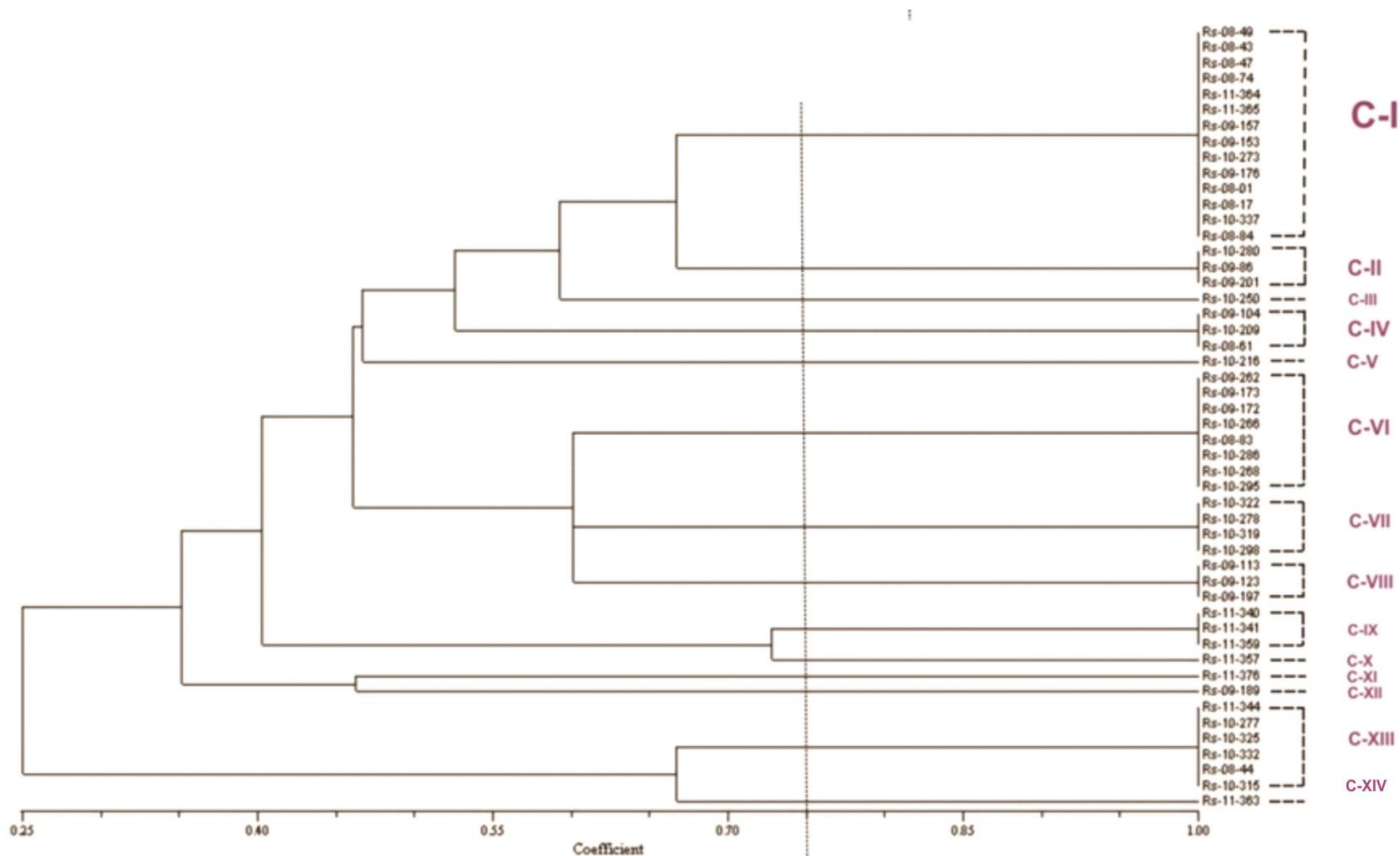


Fig. 2.15 Dendrogram showing the relationship among 50 *R. solanacearum* isolates from India based on rep-PCR using ERIC primers. Similarities and clustering (NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA) were calculated by using the Dice coefficient and UPGMA showing phylogenetic relationships.

R. solanacearum isolates viz. Rs-08-01, Rs-08-17, Rs-08-43, Rs-08-47, Rs-08-49, Rs-08-74, Rs-09-84, Rs-08-157, Rs-10-273 produced unique pattern in BOX-PCR (Table 2.5; P2) and in ERIC-PCR (Table 2.7; P1). Similarly, Rs-11-340 and Rs-11-341 also produced identical fingerprints in BOX-PCR (P5) and ERIC-PCR (P5).

Isolates in different BOX PCR and ERIC PCR clusters exhibited different host ranges (Table 2.6; 2.8). Variations were observed in the pathogenic properties of the isolates clustering together infecting the eggplant, tomato and chilli hosts. Isolates grouping together in BOX-PCR clusters I, V and VI showed many differences. Similarly, in case of isolates comprising ERIC PCR clusters I, II, III, IV, VI and VIII also displayed variations. This again proves that there was no correlation between the pathogenicity and the fingerprint pattern.

Since all the phylotype I isolates of this study showed identical PCR-RFLP profiles, the isolates were further characterized using Rep-PCR technique using BOX (Norman *et al.*, 2009; Horita *et al.*, 2010; Stevens and Elsas, 2010; Xue *et al.*, 2010; Ivey *et al.*, 2007) and ERIC primers (Horita and Tsuchiya, 2000; Norman *et al.*, 2009; Kumar *et al.*, 2004). Changes in the Rep-PCR fingerprint patterns take place due to polymorphism, rearrangements, recombination or acquisition of foreign DNA (Ishii and Sadowsky, 2009).

BOX-PCR is regarded as fast, highly resolving and reproducible PCR-based technique suitable to compare large numbers of phylogenetically related strains. Most of the strains analyzed were isolated recently (2008 upto 2011), this study provided important information on the current distribution patterns of the pathogen. Both the techniques (BOX and ERIC-PCR) used for genetic analysis were not useful in classifying the

isolates in the same cluster. However, isolates were classified into major clusters based on the geographical origin when BOX or ERIC analyses were conducted separately. Jaunet and Wang (1999) could not correlate biovars or geographic origin of tomato isolates of *R. solanacearum* to UPGMA clusters derived from Rep-PCR. Khakvar *et al.* (2008) used BOX PCR fingerprinting but could not distinguish various biovars from Malaysia. Norman *et al.* (2009) distinguished biovar 3 from biovars 1 and 2 using rep-PCR whereas in this study it could not differentiate between the biovar 3 and biovar 6 isolates. Horita and Tsuchiya (2001) successfully divided biovar N2 isolates into two distinct groups, one consisting of potato isolates belonging to Race 3, and the other consisting of tomato, eggplant, pepper, and tobacco isolates on basis of their rep-PCR fingerprints. Further, Horita *et al.* (2005) reported that each primer set (REP, ERIC and BOX) gave distinct fingerprint patterns among biovar N2 isolates which differed in geographical origin. However, the results of this study showed that no correlation was found based on the host, race, biovar or geographical origin as such.

From this study it can be assumed that major clusters differentiate the isolates based on geographical location to certain extent only. Eggplant isolates from Goa were highly diverse as unique fingerprints were generated in BOX and ERIC-PCR.

2.3.7.4. Sequencing of genes

10 isolates representing from the entire collection of isolates used under the study were selected based on host of origin, geographic location, and the greatest difference between similarity coefficients within BOX clusters.

The partial sequences genes (*mutS*, *ppsA*, *adk*, *gapA*, *egl*, *fliC*, *pglA*) of 10 Phylotype I isolates from India (Goa, Maharashtra, Orissa) (Table 2 D) were compared to the

published sequences of the reference strains within the four phylotypes for phylogenetic analysis to construct the phylogenetic trees using Neighbour Joining (NJ) algorithm (Figs. 2.16 to 2.22).

NJ trees from *egl* gene sequences showed discrete demarcation between the four phylotypes with high bootstrap values (Fig. 2.16). The isolates formed 2 groups, in which 5 of the isolates (eggplant and chilli host) showed 100% similarity within the sequences analyzed and also with two reference strains (Rs-08-11 and Rs-08-55) used for comparative analysis from India. Although Rs-09-94 and Rs-11-363 belonged to different hosts and states from India they showed 100% similarity with each other. Phylotype IV reference strains appeared in close relation with isolates under this study compared to the other phylotypes. Sequevar numbers were also assigned to 3 isolates viz. Rs-09-83, Rs-10-325 and Rs-10-332 from Goa with sequevar numbers 48, 47 and 48 respectively. Remaining isolates from Goa (n=4), Maharashtra (n=2) and Orissa (n=1) constituted unknown sequevars. Ginger isolate from Kerala and eggplant isolate from Andaman showed slight variation in their sequences.

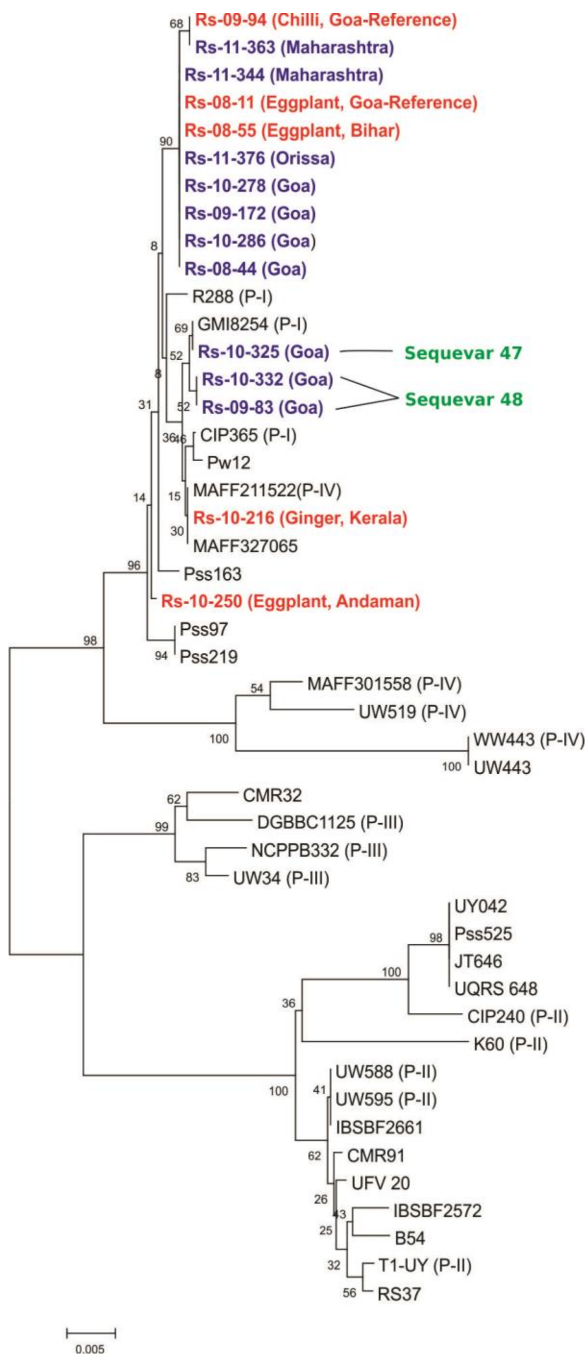


Fig. 2.16 Phylogenetic neighbour-joining tree based on the partial *endoglucanase* (*egl*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR, Research Complex for Goa. And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥ 100% are indicated at each node. The scale bar represents 5 nucleotide substitutions per 1000 nucleotides.

When *adk* gene was analyzed it was observed that 10 isolates formed two groups with less degree of variation. Six isolates showed similar gene sequences and were 100%

similar with Rs-08-55 isolate from Bihar. And the second group (Rs-10-332, Rs-10-325) exhibited identical sequences with reference strains GMI1000, GMI8254 and with ginger isolate Rs-10-216 from India. Phylotype III strains were closely related to Phylotype I strains (Fig. 2.17).

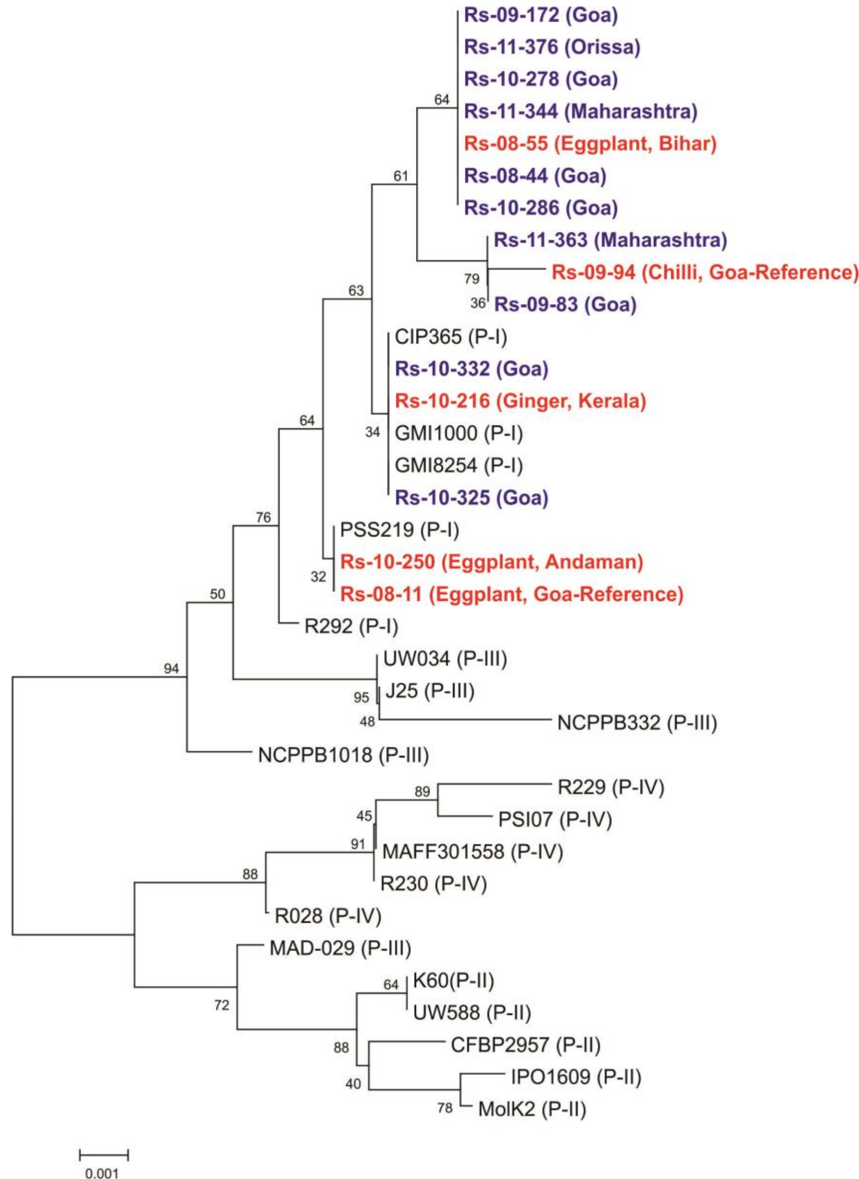


Fig. 2.17 Phylogenetic neighbour-joining tree based on the partial *adenylate kinase* (*adk*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa. And the reference strains in black were obtained from GenBankdatabase.The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥ 100% are indicated at each node. The scale bar represents 1 nucleotide substitutions per 1000 nucleotides.

All the isolates under this study and the reference isolates from India showed similar *fliC* sequences and differed slightly from GMI1000 reference strain of phylotype I. Interestingly, reference strain Phylotype IV UW521 showed 100% identical sequence with GMI1000. No distinct grouping among the phylotypes was observed. This proves that this gene is highly conserved and is less likely to get diversified (Fig. 2.18).

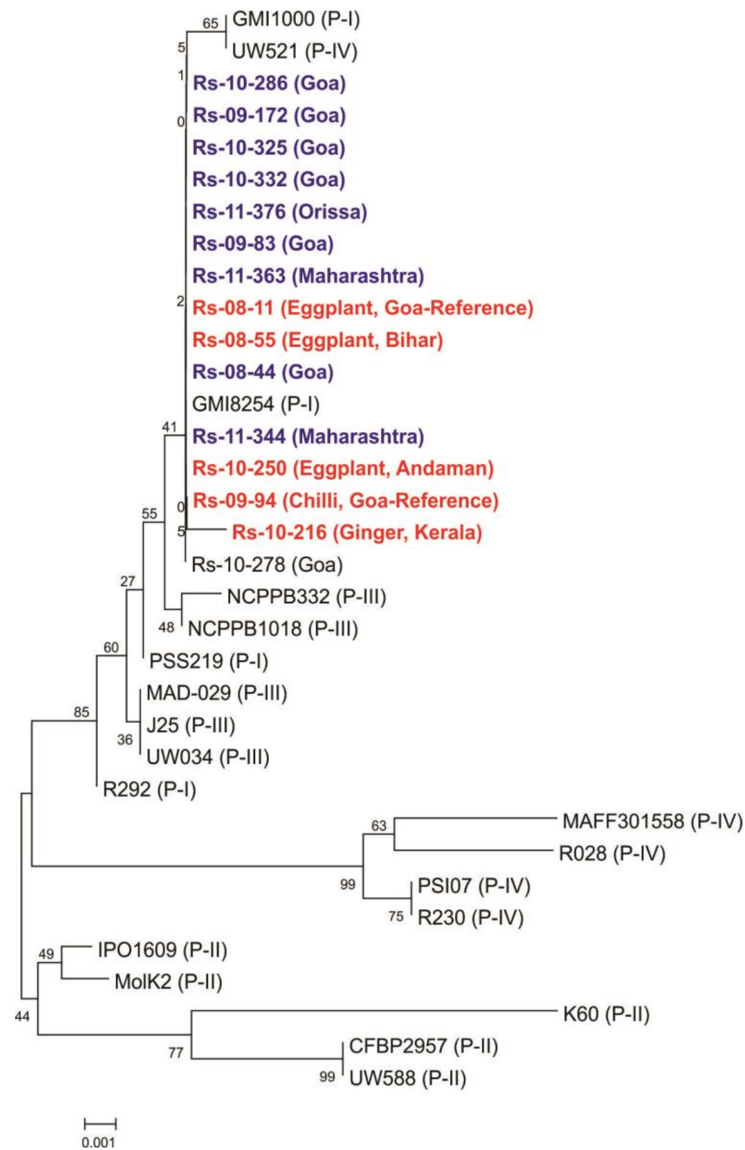


Fig. 2.18 Phylogenetic neighbour-joining tree based on the partial *flagellin protein* (*fliC*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa. And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥ 100% are indicated at each node. The scale bar represents 1 nucleotide substitutions per 1000 nucleotides.

gapA analysis generated 2 groups, one group constituted 8 isolates along with GMI1000 showed identical sequences (Fig 2.19) and in second group, 2 isolates clubbed with ginger isolate (India) and one eggplant isolate (Goa). Here again the phylotypes displayed

clear separation within the reference strains. Phylotype III was more closely associated with phylotype I isolates followed by phylotype IV and II strains.

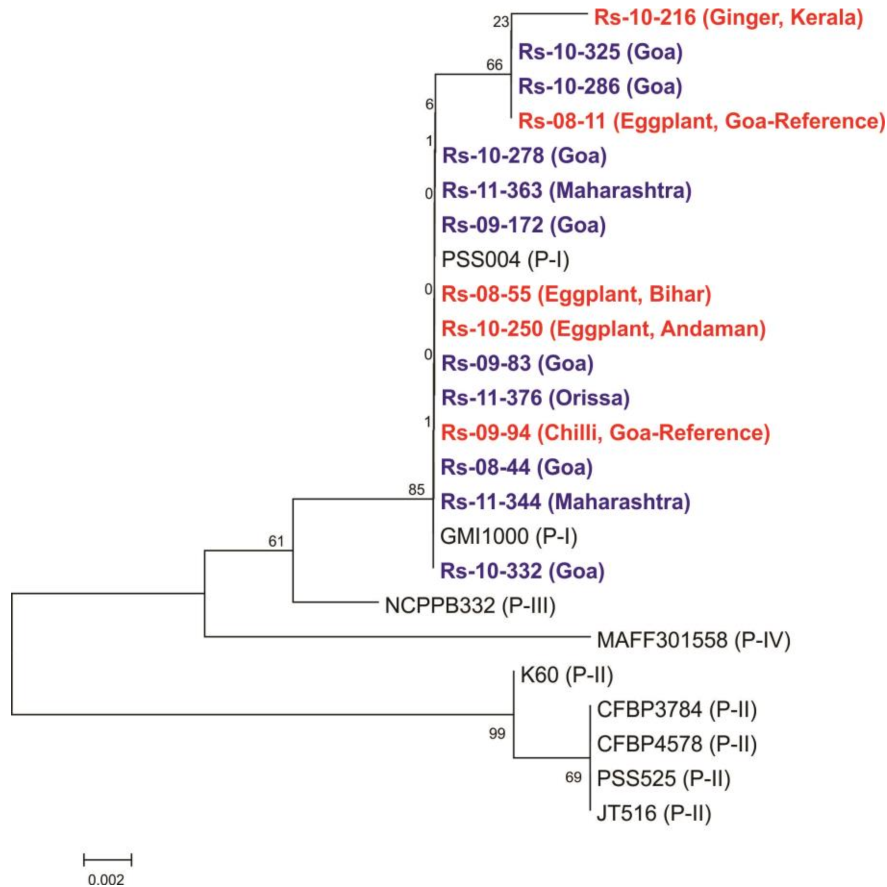


Fig. 2.19 Phylogenetic neighbour-joining tree based on the partial Glyceraldehyde-3 Phosphate dehydrogenase oxidoreductase (*gapA*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa. And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥100% are indicated at each node. The scale bar represents 2 nucleotide substitutions per 1000 nucleotides.

When *mutS* gene was analyzed, all the isolates except one (Rs-11-363) under this study clustered together in a single group along with other reference *R. solanacearum* strains (Fig. 2.20). Rs-11-363 was 100% identical with a chilli isolate from India (Rs-09-94). Phylotype IV reference strain MAFF301558 was closely related to all the phylotype I isolates from India.

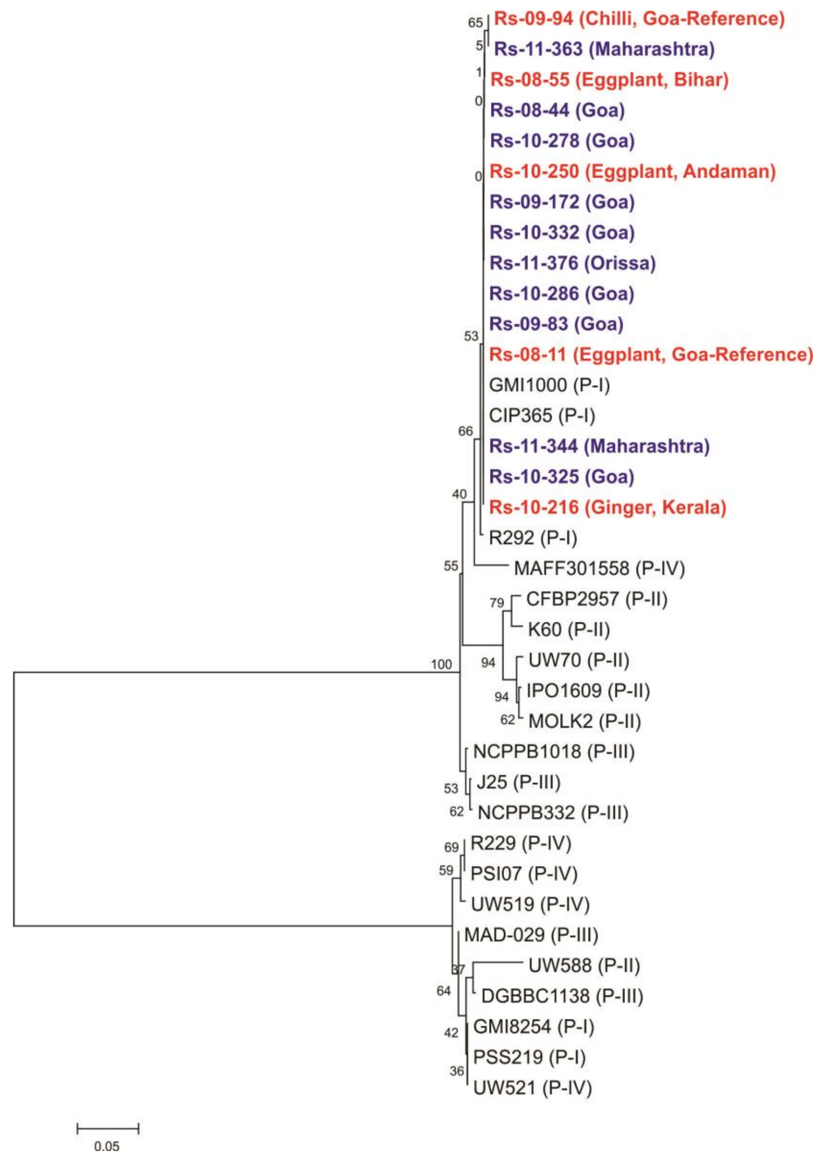


Fig. 2.20 Phylogenetic neighbour-joining tree based on the partial DNA repair gene (*mutS*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa. And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥100% are indicated at each node. The scale bar represents 5 nucleotide substitutions per 100 nucleotides.

Five of the isolates from Goa, Maharashtra and Orissa collectively showed 100% similarity in *ppsA* sequence with a reference strain (Rs-08-55) from Bihar (Fig. 2.21).

However, isolates from Goa within themselves showed slight differences and formed sub groups. Phylotype IV was closely related to Phylotype I isolates.

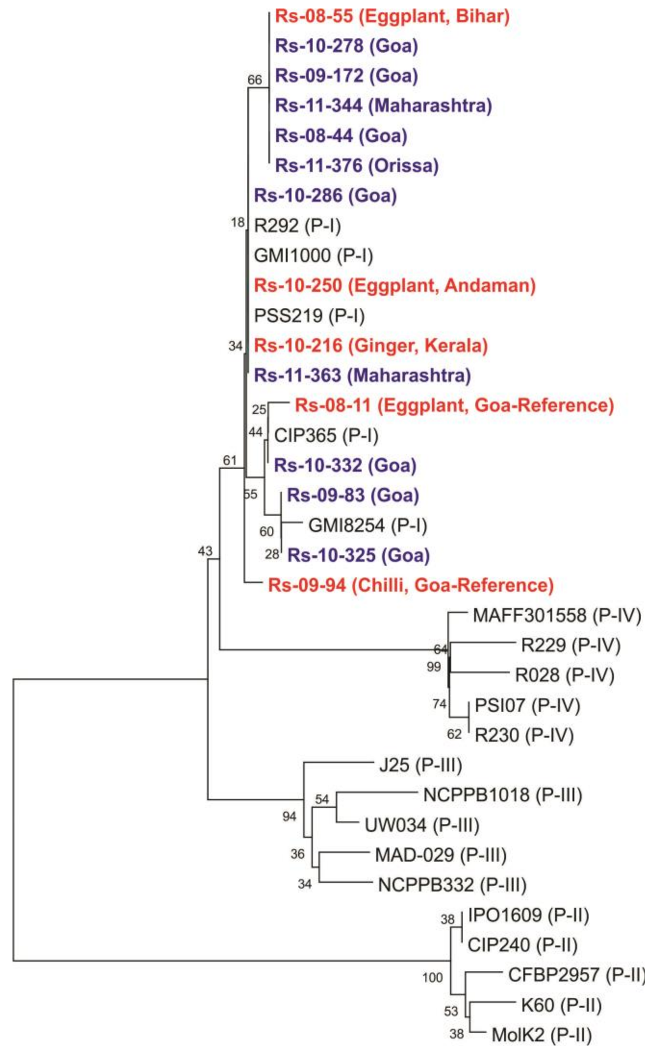


Fig. 2.21 Phylogenetic neighbour-joining tree based on the partial Phosphoenol pyruvate synthase *ppsA* gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa.And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values ≥100% are indicated at each node.

In *pglA* gene analysis, 7 isolates clustered together with a reference strain Rs-09-94 isolated from chilli host from Goa. Surprisingly, it was observed that GMI1000 was

distantly related to the collection of isolates under this study and Phylotype IV strain (UW 551) was closely related (Fig. 2.22).

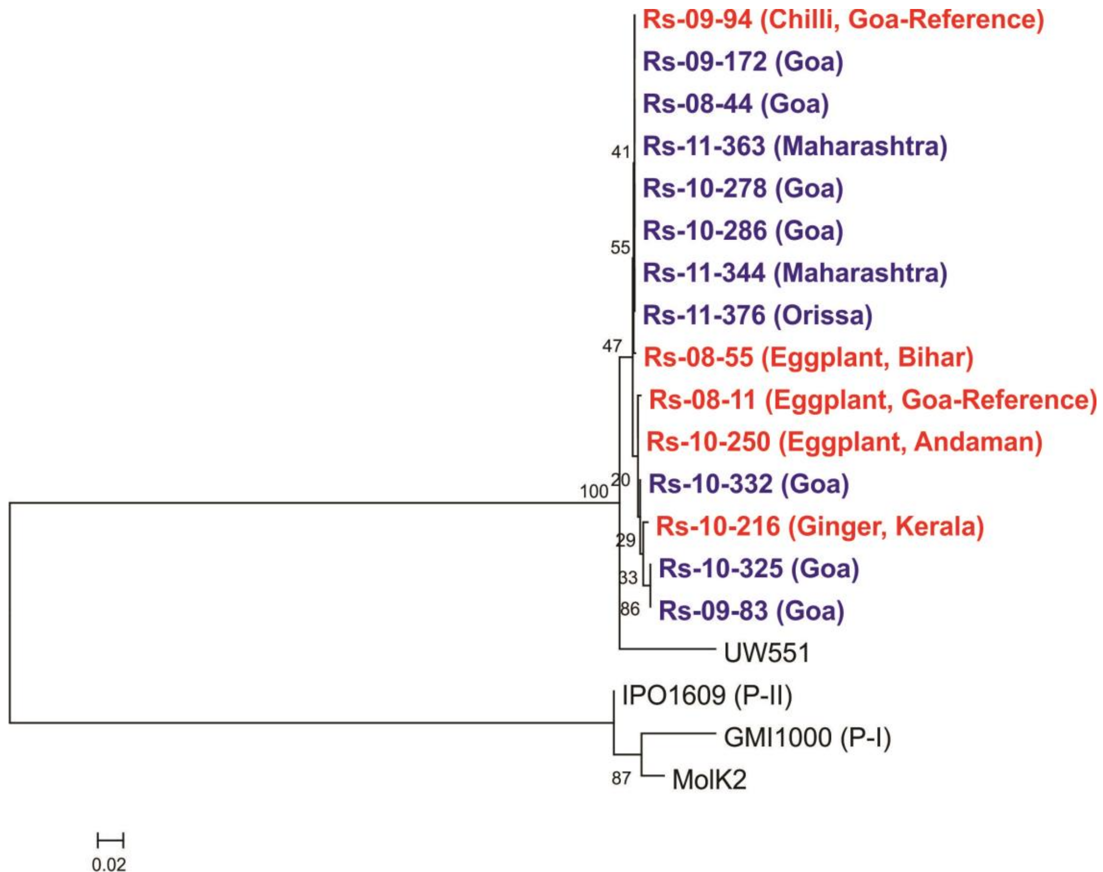


Fig. 2.22 Phylogenetic neighbour-joining tree based on the partial Polygalacturonase (*pglA*) gene sequences of strains from India and reference strains for the *R. solanacearum* representing all the phylotypes. The isolates marked in blue represent the isolates taken under this study; the isolates marked red were procured from ICAR Plant pathology laboratory, ICAR Research Complex for Goa. And the reference strains in black were obtained from GenBank database. The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values≥ 100% are indicated at each node. The scale bar represents 2 nucleotide substitutions per 100 nucleotides.

From all these results it could be concluded that there is no discrete parameter based on host, geographic region, biovar, year of isolation of pathogen or the host that the phylotype I isolates India can be classified.

Further, all the 6 genes (*mutS*, *ppsA*, *adk*, *gapA*, *egl*, *fliC*) were aligned based on their gene alignment in GMI1000 genome and concatenated sequences of the 10 isolates were

again analyzed using Neighbour Joining (NJ) algorithm. The phylogenetic relationships of these 10 isolates were compared with Phylotype I strains Rs-09-161 and Rs-10-244 (sequences obtained from Genebank) along with GMI1000. The 5 isolates viz. Rs-08-44, Rs-09-172, Rs-10-278, Rs-11-344, Rs-11-376 were collected in different years from different places of India, yet results clearly proved that they were 100 % identical with the Indian strain Rs-09-161 isolated from eggplant host (Ramesh *et al.*, 2014a). Rs-10-286 and Rs-11-363 were found to be closely related. Three isolates Rs-10-332, Rs-10-325 and Rs-09-83 were closely related to the reference strain Rs-10-244 from India (Fig. 2.23).

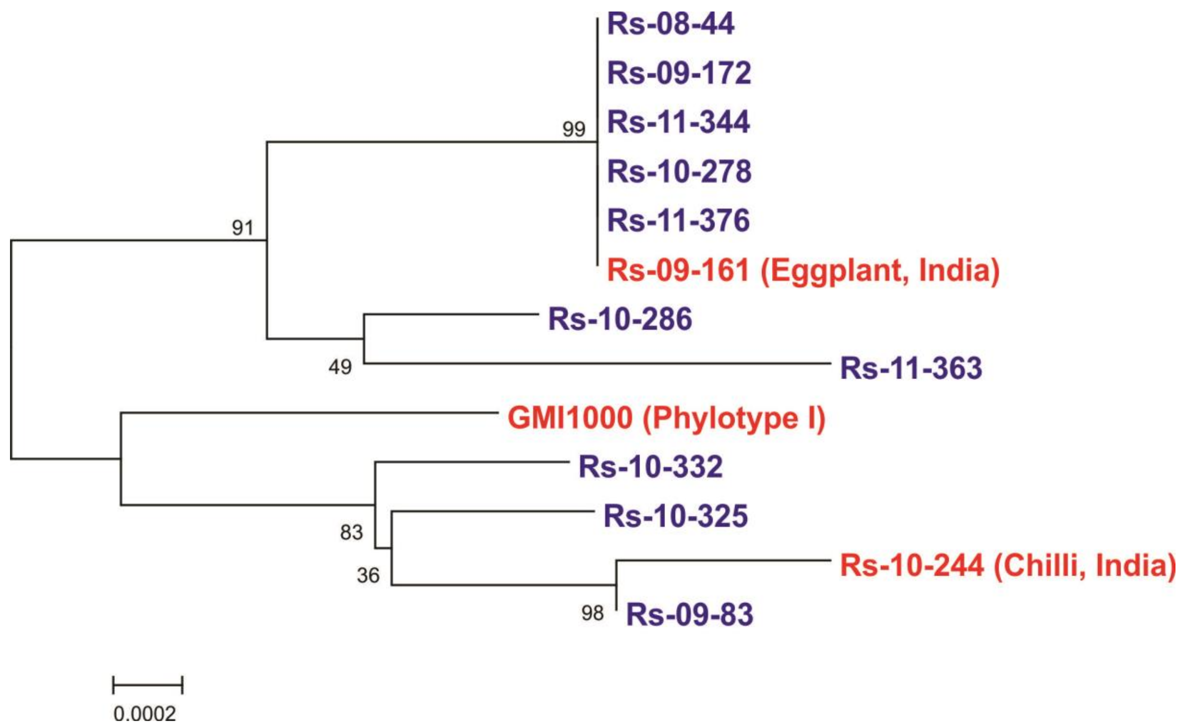


Fig. 2.23 Phylogenetic neighbour-joining tree based on the concatenated set of 6 gene sequences of 2 reference *R. solanacearum* strains from India and GMI1000 for representing phylotype I strains. The isolates marked in blue represent the isolates taken under this study; the 2 strains from India marked red were taken from Ramesh *et al.* (2014). The number at each node is the bootstrap value (1,000 resamplings), and significant bootstrap values≥ 100% are indicated at each node. The scale bar represents 2 nucleotide substitutions per 10000 nucleotides

The endoglucanase sequence analysis is powerful tool in analyzing the genetic relationship among *R. solanacearum* strains (Castillo and Greenberg 2007; Jeong *et al.*, 2007; Ivey *et al.*, 2007; Liu *et al.*, 2009; Toukam *et al.*, 2009; Nouri *et al.*, 2009; Villa *et al.*, 2005), as it encodes for a plant cell wall degrading enzyme which is directly involved in pathogenesis (Liu *et al.*, 2005). Xu *et al.* (2009) has earlier reported the existence of sequevar 48 strains belonging to Race 5, biovar 5 from china that was isolated from the mulberry host. But this study has revealed the presence of this sequevar from Goa isolated from eggplant and tomato host belonging to Race 1, biovar 3. Three isolates were assigned with 2 sequevar numbers and the remaining was reported to be new unknown sequevars. This study is in agreement with the recent report presented by Ramesh *et al.*, (2014b) on the existence of *R. solanacearum* isolates with unknown/newer sequevars amongst the Indian strains. Likewise, Sagar *et al.* (2014) characterized the genetic diversity amongst the *R. solanacearum* isolates by *egl* sequencing.

Incongruent phylogenies from partial sequences of individual genes (except *fliC*) were observed within and across phylotypes which were taken under this study. NJ trees from *egl*, *adk*, *gapA*, *pglA* and *ppsA* gene sequences were in general agreement, showing a similar branching pattern clearly partitioning the four phylotypes as previously described in the literature.

This suggests that *R. solanacearum* is a highly diverse species (Poussier *et al.*, 2000) and the genetic variation, extent of recombination, natural selection and population divergence are major factors responsible for this relatively high diversification of the pathogen (Castillo and Greenberg, 2007). All these data indicates that the *fliC* gene might

not be suitable for classifying *R. solanacearum* strains in the same phylotype, at least in phylotype I.

2.3.7.5. Multiple Locus Sequence Typing (MLST) of *R. solanacearum* isolates

Since vast diversity was found among the phylotype I isolates, MLST analysis of the selected 10 isolates (Table 2 D) was carried out to investigate aspects of the population genetics and evolution of the *R. solanacearum* species. Combination of genes would in a way help the researchers classify *R. solanacearum* strains at a much higher resolution as the genes will evolve in differently by accumulating new alleles; therefore multi locus sequence typing of 6 genes (*mutS*, *ppsA*, *adk*, *gapA*, *egl*, *fliC*) was studied. These were aligned based on the GMI1000 genome alignment as all the isolates were phylotype I. Sequences of 6 genes were analysed using different softwares so as to define haplotypes by nucleotide sequencing, rather than indirectly from the electrophoretic mobility of their gene products. Sequence data for Phylotype I reference strains was also analyzed along with these 10 isolates for comparative study (Table 2 H). A few important phylogenetic parameters were recorded for the sequence data from all the isolates. Nucleotide diversity (Table 2.9) was in the range of 0.00041-0.00358 across all the genes when all the 10 isolates were compared with each other. It was highest in case of *egl* gene (0.00358) and lowest in case of *mutS* gene (0.00041). There was no diversity when *fliC* gene sequences were compared (Pi=0). Maximum number of polymorphic sites were present in *egl* sequences (6 sites), *gapA* (5 sites) and 3 sites each for *adk* and *ppsA* genes sequences. Only 1 site was detected for *mutS* gene, but none in case of *fliC* gene.

Table 2.9 Genetic diversity estimators of the partial gene sequences of *R. solanacearum* isolates.

Domain	Region	*n*	*Sites*	*S*	*Eta*	*Hap*	*Hd*	*VarHd*	*Pi*	*ThetaNuc*	*k*	*ThetaG*	Tajima D	Sig D	FuLiD *	Sig D	FuLiF*	Sig F	FuFs
Phylotype I reference																			
mutS	1-463	3	463	0	0	1	0	0	0	n.a.	0	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
ppsA	464-1093	7	630	6	6	5	0.791	0.00741	0.00349	0.0029	2.1978	1.8867	0.5919		1.2881	#	1.2604		0.31
adk	1094-1513	7	420	6	6	5	0.791	0.00741	0.0046	0.0044	1.9341	1.8867	0.0901		1.2881	#	1.1092		-0.008
gapA	1514-2287	3	774	2	2	3	0.8	0.01481	0.00138	0.0011	1.0667	0.8759	1.0319		1.2797		1.2748		-0.082
egl	2289-2928	11	641	12	12	10	0.935	0.00063	0.00627	0.0051	4.0173	3.2919	0.7722		1.4627	*	1.4644	#	-1.29
fliC	2929-3237	6	309	3	3	4	0.727	0.01178	0.00353	0.0032	1.0909	0.9934	0.3219		1.1049		1.0255		-0.433
CS	1-3237	3	3237	17	17	3	0.8	0.01481	0.0028	0.0023	9.0667	7.4453	1.3508		1.6835	**	1.7539	*	4.542
Isolates under this study																			
mutS	1-463	10	463	1	1	2	0.189	0.01169	0.00041	0.00061	0.1895	0.2819	-0.5916		0.6495		0.3673		-0.097
ppsA	464-1093	10	630	3	3	4	0.695	0.00618	0.00207	0.00134	1.3053	0.8456	1.4518		1.0065		1.2976		0.627
adk	1094-1513	10	420	3	3	3	0.589	0.00857	0.00281	0.00201	1.1789	0.8456	1.0529		1.0065		1.172		1.627
gapA	1514-2287	10	774	5	5	6	0.737	0.00868	0.00237	0.00182	1.8316	1.4093	0.9167		1.1864		1.2817		-0.515
egl	2289-2928	10	641	6	6	4	0.611	0.01068	0.00358	0.00264	2.2947	1.6912	1.1372		1.2487		1.4059		2.222
fliC	2929-3237	10	309	0	0	1	0	0	0	n.a.	0	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
CS	1-3237	10	3237	18	18	6	0.737	0.00868	0.0021	0.00157	6.8	5.0737	1.2801		1.5602	**	1.7175	*	4.092
All Phylotype I strains																			
mutS	1-463	13	463	1	1	2	0.148	0.00788	0.00032	0.00057	0.1477	0.2621	-0.7139		0.6121		0.2883		-0.317
ppsA	464-1093	17	630	6	6	6	0.777	0.00178	0.00278	0.00233	1.754	1.4674	0.5408		1.2045		1.1706		0.18
adk	1094-1513	17	420	6	6	6	0.742	0.00252	0.00414	0.00349	1.7398	1.4674	0.5138		1.2045		1.1611		0.155
gapA	1514-2287	13	774	5	5	7	0.763	0.00552	0.00219	0.00169	1.6985	1.3103	0.8395		1.1581		1.2362		-1.206
egl	2289-2928	21	641	13	13	11	0.855	0.00156	0.00581	0.00471	3.7259	3.0212	0.7212		1.5076	*	1.4734	#	-0.497
fliC	2929-3237	16	309	3	3	4	0.339	0.01081	0.00151	0.00241	0.4677	0.7449	-0.86		0.9418		0.4869		-1.311
CS	1-3237	13	3237	22	22	8	0.775	0.00601	0.0023	0.00178	7.4585	5.7653	1.0627		1.6351	**	1.7077	*	3.341

*Neutrality tests conducted using 3 methods for determining the significance ;Statistical significance: *: P< 0.05; **: P< 0.02; #: NS, 0.10 P> 0.05; n.a- not analyzed; n.d.- not determined; *S*-Number of polymorphic (segregating) sites; *Eta*-Total number of mutations, Hap-Number of Haplotypes; *hd*-Haplotypes (gene) diversity; varHd- Variance in haplotype diversity; *Pi*-Nucleotide diversity; k-Average number of nucleotide differences; CS-Concatenated set; Minimum number of recombination events was calculated for all the genes, it was zero, except for *gapA* gene it showed 1. For CS it was 4.

Haplotype numbers for each of the 6 genes ranged from 1 (*fliC*) to 6 (*gapA*) as presented in Table 2.10. This was also proved by the fact that the maximum haplotype diversity (Hd) was recorded for *gapA* (0.737) gene followed by *ppsA* (0.695) gene. No diversity was observed for *fliC* gene which exhibited a single unique haplotype.

5 isolates viz. Rs-08-44, Rs-09-172, Rs-10-278, Rs-11-344 and Rs-11-376 were 100% identical to each other and belonged to haplotype 1 as shown in the Table 2.10. All were isolated from eggplant host. Their sequevars are yet to be identified. Isolate Rs-10-286 was closely related to the set of 5 identical isolates. Rs-10-332 and Rs-10-325 from Goa were closely related to each other although they belonged to different sequevars compared to others. The frequency of mutations was more in *egl* as compared to other genes. No mutation was observed in *fliC* gene. Only one recombination event was detected in *gapA* gene sequences between 63 and 663 site. Remaining 6 genes appeared to be recombination free. More than 98% of the sequence was conserved in all the genes, *fliC* displaying 100% identical sequence in all the isolates under study.

Table 2.10 Identification of haplotypes of 10 isolates of *R. solanacearum* and the concatenated set based on 6 genes.

Gene s		Isolates/host									
	No. of haplotypes identified	Rs-08-44 (E)	Rs-09-172 (E)	Rs-10-278 (E)	Rs-11-344 (E)	Rs-11-376 (E)	Rs-10-286 (E)	Rs-10-332 (C) *Seq 48*	Rs-10-325 (E) *Seq 47*	Rs-11-363 (E)	Rs-09-83 (T) *Seq48*
mutS	2	1	1	1	1	1	1	1	1	2	1
ppsA	4	1	1	1	1	1	2	4	3	2	3
adk	3	1	1	1	1	1	1	2	2	3	3
gapA	6	1	1	1	1	1	2	4	3	6	5
egl	4	1	1	1	1	1	1	2	3	4	2
fliC	1	1	1	1	1	1	1	1	1	1	1
CS	6	1	1	1	1	1	2	3	4	5	6

E- Isolated from eggplant; C- Isolated from Chilli; T- Isolated from tomato
CS-Concatenated set; *Seq*-sequevar; Unknown sequevars for other isolates

All the isolates belonged to phylotype I and therefore all the sequences were concatenated and arranged. The length of this concatenate was 3237 bp. This set of concatenated genes was also analyzed together using DnaSP 5.0 software for all the isolates. Average number of nucleotide difference was calculated to be 6.8. It generated 6 haplotypes and recorded the haplotype diversity of 0.737. A total of 4 recombination events were detected in the concatenated data set between sites (748, 1231) (1417, 1576) (1576, 2176) (2176, 2467). This distribution of recombination events suggests that the recombination has taken place in genes between *ppsA* and *adk*; *adk* and *gapA*, within *gapA* and *egl*. *mutS* and *fliC* genes appeared as recombination free.

Splitstree4 software (Huson and Bryant, 2006) was used to draw neighbor net reconstruction (Fig. 2.24) to determine the evolutionary relationship between the 10 isolates. The Neighbor Net displays 99.8% of the data, almost all the data is represented as 20 splits. Numbers of constant sites were 3215 of the total 3237 nucleotide sites. The estimated invariant sites were 0.00694. This splits tree was constructed based on 1000 bootstrap analysis. All the isolates appear to form reticulate network which can be clearly related to incongruent phylogenies from partial sequences of individual genes suggests that there are recombinational events within the phylotype. The results are in agreement with a section of work on phylotype I reported by Wicker *et al.* (2012) in his recent article on multilocus sequence analysis (MLSA) of *R. solanacearum*.

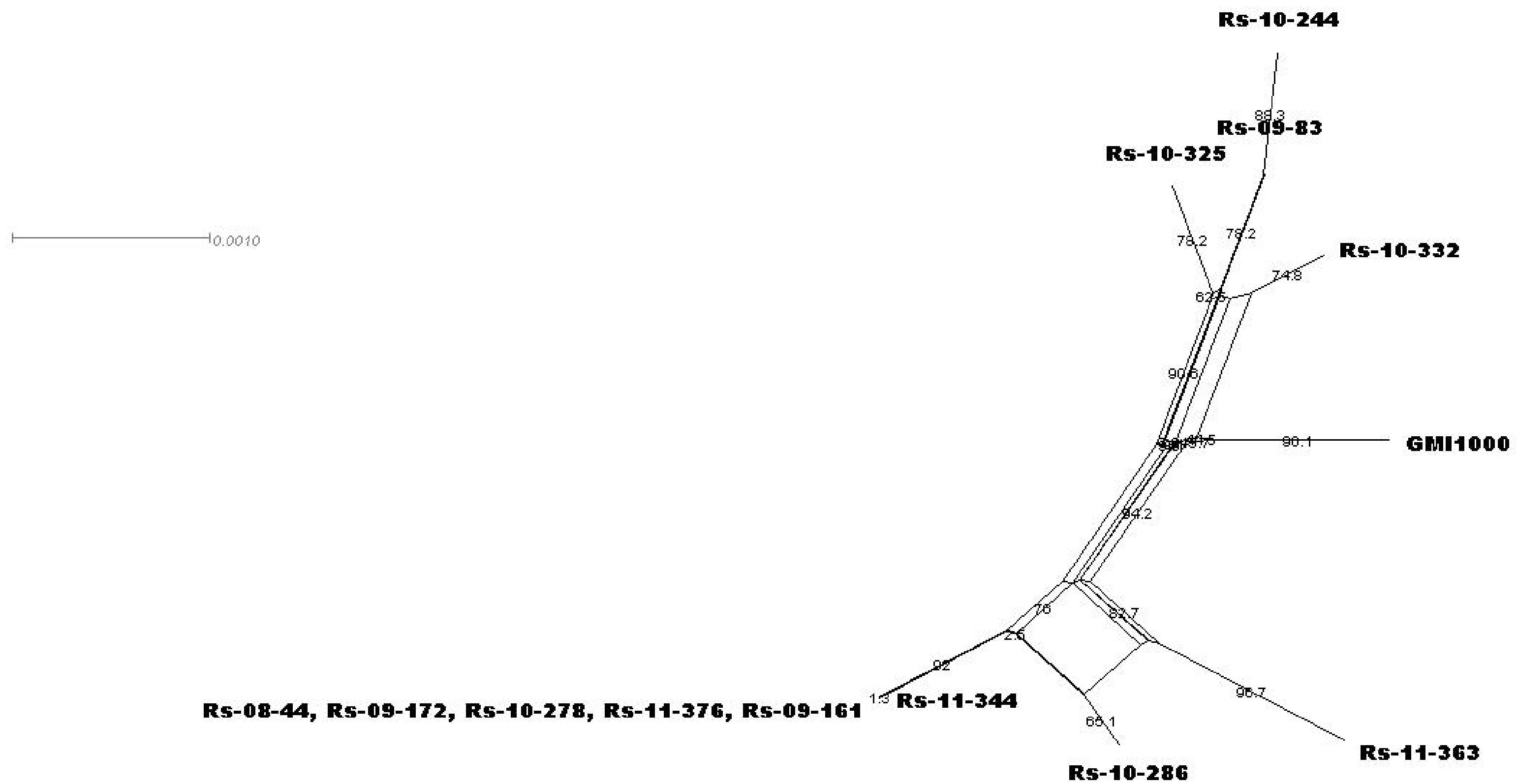


Fig. 2.24 Splitstree designed based on Neighbor Net analysis of aligned sequence data for visualizing the evolutionary relationships between the 10 isolates. The numbers on the nodes are bootstrap values. The scale bar represents 1 nucleotide substitutions per 1000 nucleotides

When all the 6 genes were compared together for determining the haplotypes within the 10 isolates, interesting results were observed. A total of 6 haplotypes were detected based on the concatenated set of all the genes. 5 isolates from Goa, Maharashtra and Orissa group into haplotype 1 belonging to eggplant host. Remaining isolates belonged to a unique haplotype each. From the results obtained in this study it is observed that there is a possibility that high levels of recombination took place at the local population even with little variation in mutation rates. Recently, Guidot *et al.* (2009) have reported that *R. solanacearum* can become naturally competent although, the factors responsible for this behavior are unknown. In spite of the mechanisms which can block this process (Mercier *et al.*, 2007) of DNA uptake from environment, the external foreign DNA successfully integrates into pathogen genome and hence the variations in the genetic makeup.

The results of this study therefore agree with the findings of Perez-Losada *et al.* (2006) that recombination and mutation generates the genetic variation and these factors can potentially create new variants. The combined effect of mutation, recombination, genetic drift and selection pressure measures the number of possible allelic states as well as their frequency distribution within the population of *R. solanacearum*. Mutation rates at an individual gene locus are thought to be partially intrinsic to that locus, but they are also a function of more global mutational processes (Keim *et al.*, 2004). Such changes may take place under either neutral or diversifying selection thereby increasing diversity, or under directional selection which leads to fixation of particular changes within the population (Lobry and Sueoka, 2002).

R. solanacearum is known to possess a high ability for recombination (Coupat *et al.*, 2008), its broad host range, its readiness to adapt to environmental conditions (Wicker *et*

al., 2012) and pathogenic characteristics are in favor of phylotype I isolates to have a high evolutionary potential (Mc Donald and Linde, 2002). With evolutionary time and with number of mutations, genetic heterogeneity develops and this eventually allows the individual lineages to be distinguished.

It would be very interesting to know which genes are responsible for this genetic evolution. These findings and investigations in the genomic analysis of the *R. solanacearum* pathogen have focused on some previously obscure aspects of this pathogen's inner workings. This type of studies will help ther esearchers to provide innovative tools and strategies to control *R. solanacearum* wilt diseases.

2.3.7.6. Nucleotide sequence accession numbers

The nucleotide sequence data of the 10 *R. solanacearum* isolates have been deposited into the GenBank database and the accession numbers are obtained (Table 2.11). *egl* gene sequences under accession numbers KF920632-KF920641; *pglA* gene sequences under accession numbers KF920642-KF920651; *mutS* gene sequences under accession numbers KF920652-KF920661; *adk* gene sequences under accession numbers KF920662-KF920671; *gapA* gene sequences under accession numbers KF920672-KF920681; *ppsA* gene sequences under accession numbers KF920682-KF920691 and *fliC* gene sequences under accession numbers KF920692-KF920701.

Table 2.11 List of Nucleotide sequence accession numbers submitted to GenBank

No.	Isolate	Place	Crop	Gene bank accession numbers						
				egl	***pglA***	***mutS***	***adk***	***gapA***	***ppsA***	***fliC***
1	Rs-08-44	Goa	Eggplant	KF920632	KF920642	KF920652	KF920662	KF920672	KF920682	KF920692
2	Rs-09-172	Goa	Eggplant	KF920633	KF920643	KF920653	KF920663	KF920673	KF920683	KF920693
3	Rs-10-278	Goa	Eggplant	KF920634	KF920644	KF920654	KF920664	KF920674	KF920684	KF920694
4	Rs-10-286	Goa	Eggplant	KF920635	KF920645	KF920655	KF920665	KF920675	KF920685	KF920695
5	Rs-10-325	Goa	Eggplant	KF920636	KF920646	KF920656	KF920666	KF920676	KF920686	KF920696
6	Rs-10-332	Goa	Chilli	KF920637	KF920647	KF920657	KF920667	KF920677	KF920687	KF920697
7	Rs-09-83	Goa	Tomato	KF920638	KF920648	KF920658	KF920668	KF920678	KF920688	KF920698
8	Rs-11-344	Maharashtra	Eggplant	KF920639	KF920649	KF920659	KF920669	KF920679	KF920689	KF920699
9	Rs-11-363	Maharashtra	Eggplant	KF920640	KF920650	KF920660	KF920670	KF920680	KF920690	KF920700
10	Rs-11-376	Orissa	Eggplant	KF920641	KF920651	KF920661	KF920671	KF920681	KF920691	KF920701

Chapter III

Pathogenicity and virulence factors of *R. solanacearum* infecting eggplant

3.1. Introduction

R. solanacearum, a soil-borne bacterium, known to cause bacterial wilt in numerous plant species, is widely accepted for the study of pathogenicity in plants (Hayward, 1991). This pathogen is a very challenging bacterium which has destroyed the fields because of its extreme aggressiveness, geographic distribution, and wide host range (Champoiseau *et al.*, 2009).

As previously reported by Araud-Razuo *et al.*, (1998) the pathogen invades plant roots from the soil through wounds or natural openings where secondary roots emerge, inhabits the vascular tissue of its host by colonizing the root cortex and vascular parenchyma by multiplying itself to 10^9 CFU g^{-1} of host tissue. In the later stages of infection, it requires highly specialized process of interacting genes and protein products of the pathogen as well as of the plant. This eventually leads to massive amounts of bacterial cells inside the plant vascular tissue. Symptoms include chlorosis, stunting and wilting resulting in the death of the host.

Many factors contribute to this overall infection process. These include Cell-Wall-Degrading Enzymes (CWDEs) (Huang and Allen, 2000; Liu *et al.*, 2005) viz. polygalacturonase (PG) and endoglucanase (EG) (Schell *et al.*, 1988; Saile *et al.*, 1997), flagella-driven swimming motility and type IV pili-driven twitching motility (Tans-Kersten *et al.*, 2004), extracellular polysaccharide I (EPS I) (Orgambide *et al.*, 1991; Denny, 1995; Araud-Razou *et al.*, 1998), chemotaxic behavior (Yao and Allen, 2006), the

type III secretion system (T3SS) (*Hrp* machinery) that allows the secretion and the injection of effector proteins into plant cells (Cornelis and Van Gijsegem, 2000; Gueneron *et al*., 2000) and type II secretion sytem (T2SS) (Lui *et al.*, 2005).

Interestingly, all of these virulence factors are controlled by a complex regulatory signal transduction pathway (Schell, 2000; Hikichi *et al*., 2007) that responds to both environmental signals and quorum sensing (Brito *et al*., 1999). PhcA, a regulatory protein which plays a central role in a complex regulatory cascade is mediated by the specific endogenous signal molecule, 3-hydroxypalmitic acid methyl ester (PAME) (Clough *et al*., 1997; Flavier *et al*., 1997). Although not much is understood about these virulence factors and their regulation, less is known about how *R. solanacearum* effectively adheres, colonizes and spreads in the host.

This chapter deals with the virulence of the *R. solanacearum* isolates on eggplant and production of certain important virulence factors using 3 different wild type *R. solanacearum* isolates differing in aggressiveness on the host and 2 EPS I mutant strains.

3.2. Materials and Methods

3.2.2. Virulence testing of *R. solanacearum* isolates on eggplant

3.2.2.1. Inoculation of *R. solanacearum*

Pathogenicity of the *R. solanacearum* isolates was tested and reported in Chapter II. Study on virulence of the isolates is reported in this chapter. For virulence testing, eggplant (*Solanum melongena* L.) cv. *Agassaim* seedlings were used. This variety is known to be highly susceptible. Virulence of *R. solanacearum* strains was tested by using both petiole inoculation and soil drenching inoculation methods on 25-day old eggplant seedlings. 20 days-old eggplants were transplanted into the pots containing the sterile

nursery mixture (soil: FYM: sand in ratio of 2:1:1) in green house at 30°C and inoculation of *R. solanacearum* was carried out on the 25th day.

R. solanacearum isolates were grown in 20 mL CPG broth for 48 h and was centrifuged at 10,000 rpm for 8 min at room temperature. The pellet was suspended in 100 mL of sterile 1X PBS (composition mentioned in Chapter II) and inoculum population was adjusted to 7 log CFU mL^{-1}. 10 mL of inoculum prepared in PBS was inoculated to each plant by soil drenching (Schaad *et al.*, 2001) and petiole inoculation (Saile *et al.*, 1997). Soil drenching was performed by merely pouring the culture suspension near the root zone. Whereas, petiole inoculation was performed by pricking the first petiole above the cotyledons, at 5 mm from the stem and 2 μL of inoculum was deposited onto the surface of the pricked portion. Plants inoculated with sterile 1X PBS served as negative control. Prior to inoculation plants were not watered for 24 h. 5 plants per pot in two replicates were inoculated and this experiment was repeated twice. Wilt incidence was recorded daily as percentage of leaves wilted up to 30 days time period.

3.2.2.2. Disease scoring

Percent wilt was calculated according to standard formula and Percent Severity Index (PSI) was calculated based on 0-4 disease index scale, where 0 indicates no disease, 1 indicates 1 to 25% of leaves wilted, 2 indicates 25 to 50% of leaves wilted, 3 indicates 51 to 75% of leaves wilted, and 4 indicates 76 to 100% of leaves (Schell *et al.*, 1988; Roberts *et al.*, 1988). PSI was calculated as per the formula described by Cooke (2006). PSI = Σ (scores × 100)/ (number of plants rated × maximum scale of the scores) for each scoring date.

3.2.3. Pathogenicity of non-EPS *R. solanacearum* isolates on eggplant

R. solanacearum isolates used in this experiment are Rs-08-17 (highly virulent) and a non-EPS I producer (Rs-100C). The testing was performed in 3 batches and each time the inoculum population was increased by 10 fold. Inoculation of *R. solanacearum* and the experimental set up were done as per the protocol mentioned in section 3.2.2.1 with different inoculum concentrations. In batch I, 7 log CFU mL^{-1} *R. solanacearum* was inoculated to five plants for each treatment and the entire experiment was repeated twice. In batch II, *R. solanacearum* inoculums was 8 log CFU mL^{-1} and in batch III, the higher concentration of *R. solanacearum* inoculum (9 log CFU mL^{-1}) was used for inoculating the eggplants. In all the experiments the disease incidence was monitored up to 30 DAI.

3.2.4. Histopathology studies of eggplant infected with bacterial wilt

The wilted eggplant was uprooted and washed with clean water and dried. A clean and sharp blade was taken to take thin cross sections of stem portion. These sections were placed in 0.05% safranine solution for 30 seconds and again kept in distilled water to remove the excess stain. These sections were later mounted on a clean glass slide with a coverslip and a drop of water. It was observed under 4X and 40 X magnifications of compound microscope (Olympus CX41 phase contrast microscope)

3.2.5. Study on the virulence factors of *R. solanacearum*

3.2.5.1. *R. solanacearum* isolates used under this study

Five isolates viz. Rs-08-17, Rs-09-100, Rs-09-109, Rs-100C and Rs-100D were used for studying various factors responsible for causing the infection in the host. Rs-08-17 was isolated in this study and the remaining 4 strains were obtained from Plant pathology laboratory, ICAR, Research complex for Goa, Goa.

3.2.5.2. Motility and chemotaxis Assays

3.2.5.2.1. Motility of *R. solanacearum*

R. solanacearum isolates were grown in CPG broth for 18 h at 28°C on a rotary shaker at 150 rpm. Hanging drop wet mount method was employed to study the bacterial motility under 100 X magnification of Olympus CX41 phase contrast microscope. A drop of bacterial culture was placed in the middle of a cover slip. Petroleum jelly was applied at the edges of the cover slip and the cavity slide was turned upside-down (depression area facing down) over the cover slip such that the jelly holds the cover slip to the slide and also keeps the suspension from drying out. The slide was flipped and mounted under the microscope.

Swimming and swarming motility: Swimming motility assay were performed at 28°C on freshly prepared soft agar plates (1% tryptone, 0.25% NaCl) containing 0.3% agar. Swarming motility was checked on BMM [$NH_4(SO_4)_2$, 10g L^{-1}; KH_2PO_4, 68g L^{-1}; $FeSO_4$, 2.5mg L^{-1}; 1 mL of 1 M $MgSO_4.7H_2O$, 1000 mL distilled water] and BG medium (Bacto peptone, 1%; casamino acids, 0.1%; yeast extract, 0.1%; glucose, 0.5%) containing 0.5% agar. For swimming and swarming motility, 5μL of the bacterial broth culture (8 log CFU mL^{-1}) was placed directly on the center of the agar so that the motility within the semisolid agar could be visualized and evaluated (Deziel *et al.*, 2003; Tremblay *et al.*, 2007). The diameter of the swarming motility assay was measured after 24 h of incubation. This experiment was repeated twice.

Twitching motility: Twitching motility assay was performed on 1.5% Luria Burtani agar (Tryptone, 10g L^{-1}; yeast extract, 5g L^{-1}; NaCl, 10g L^{-1}; 1000 mL distilled water) plates by stab-inoculating a single bacterial colony deep into the agar with a sterile toothpick so

that the toothpick touched the agar-dish interface as described by Murray *et al.* (2010). The plates were observed after overnight incubation under the compound microscope with 4X objective (Olympus CX41 phase contrast microscope).

3.2.5.2.2. Chemotaxis

Collection of plant root exudates: Eggplant seeds were surface sterilized by soaking the seeds in 0.1% $HgCl_2$ solution for 2 min, followed by washing them twice in sterile water for 5 min with gentle shaking. The seeds were then transferred onto 1% water agar plates (Agar, 10g L^{-1}; 1000 mL distilled water), which were incubated at 4°C overnight and then kept in a 28°C dark incubator for 3 to 5 days for germination. Twenty germinated seedlings with long roots were transferred into a sterile 5 mL glass tube containing 5 mL of sterile chemotaxis buffer (10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM $MgSO_4$) and incubated at 28°C for 24 h. The eggplant root exudates were collected, concentrated in rotary vacuum evaporator and filter sterilized through a 0.22 um filter, and stored at -80°C.

Chemotaxis assay: Chemotaxis behavior of *R. solanacearum* (Rs-08-17, Rs-100C, Rs-100D) isolates was carried out in two ways. First the assay was done in the broth and later on the semisolid medium. BMM broth with 10 mM for sugars (glucose, fructose, galactose, saccharose, trehalose, rhamnose, sucrose and xylose), 10 mM for organic acids (citric acid, malic acid, succinic acid, glucuronic acid and galacturonic acid), and 4 mM for amino acids (alanine, glycine, tryptophane and aspartate) was used for liquid assay. After 24 h incubation at 28 °C the turbidity of the culture was measured (OD_{600}); moderate growth was considered as an OD_{600} between 0.1 and 0.6, and vigorous growth as an $OD_{600} \geq 0.6$.

In BMM semisolid medium assay, 25µL of overnight grown *R. solanacearum* isolates (6 log CFU mL^{-1}) was added to 100 mL of molten BMM medium and poured into sterile Petri plates and allowed to solidify. Three wells of 5 mm diameter each were bored per plate using sterile cork borer and 35µL of root exudates was added in the wells. Controls were maintained using chemotactic buffer and 1X PBS. The plates were incubated at 28°±2°C C for 3 to 5 days. Chemotactic response was measured by the size of the halo produced around the inoculation site. Chemotaxis assays were performed in duplicate.

3.2.5.3. Siderophore Production

Chrome azurol S (CAS) plates were prepared according to Schwyn and Neilands (1987) using 60.5mg Chrome azurol S (CAS) dissolved in 50 mL distilled, deionized water and mixed with 10 mL iron (III) solution (27mg $FeCl_3 .6H_20$, 83.3µL of Conc. HCl in 100 mL of distilled water). Under stirring, this solution was slowly added to 72.9mg CTAB dissolved in 40 mL water. The resultant dark blue mixture was diluted 20-fold and autoclaved at 121°C for 15 min. The basal medium used was King's B medium. 10 mL of CAS indicator solution was added to 90 mL of molten basal medium (autoclaved) very slowly along the glass walls, with enough agitation to achieve mixing without generation of bubbles under aseptic conditions. The agar turned blue in color which was instantly poured into sterile petriplates. *R. solanacearum* isolates were grown in CPG broth for 18-24 h, later spot inoculated on the CAS agar plates and incubated at 30°C for 3 days. Positive results were detected by a color change from blue to orange after incubation. The presence of a halo around the colony indicates siderophore production and size of the halo was recorded. The assay was conducted in two replications.

3.2.5.4. Screening for Pectinase production

3.2.5.4.1. Qualitative assay: Standard pectinase concentrations were prepared using Pectinase solution from *Aspergillus niger* having 46 mg mL^{-1} (16 units mg^{-1} protein) protein at following concentrations viz. 5.0, 0.5, 0.05, 0.005 and 0.0005 mg mL^{-1}. Assay medium contained 0.5% ammonium oxalate, 0.2% sodium azide, 1% Type II agarose in 0.2 M Phosphate buffer (pH 5.3) (Appendix D.2) and 0.01% PGA sodium salt. Wells of 7 mm diameter were bored using a sterile cork borer. The cultures (Rs-08-17, Rs-09-100, Rs-09-109, Rs-100C and Rs-100D) were grown in Chatterjee's medium (Chatterjee, 1980) [KH_2PO_4, 2g L^{-1}; K_2HPO_4, 7g L^{-1}; $MgSO_4.7H_2O$, 0.1 g L^{-1}; $(NH4)_2SO_4$, 1g L^{-1}; tryptone, 10g L^{-1}; NaCl, 10g L^{-1}; and yeast extract, 5g L^{-1}, with pH adjusted to 7.0] with 1% citrus pectin for 72 h and centrifuged at 10,000 rpm for 12 min. The supernatant was later filtered through 0.45µm filter and was used in the assay medium. 40µL of pectinase standard solutions and the culture supernatants were added in the wells and were incubated at 37°C for 17 h. After the incubation period, the plates were flooded with 0.05% ruthenium red solution and incubated at 30°C for 30 min. The plates were then washed with distilled water to observe the zones.

Pectinase activity was also rechecked on a simple pectin medium (0.5% citrus pectin and 0.5% yeast extract, pH 7.0). 40µL of pectinase standard solutions and the culture supernatants were added in the wells and incubated at 37°C for 3 days. Later the plates were flooded with 1% Cetrimide solution and zones of degradation were observed and recorded. These plate assays were conducted in two replications.

3.2.5.4.2. Quantitative assay of crude pectinase

400µL of *R. solanacearum* isolates (8 log CFU mL^{-1}) was inoculated in 20mL of

pectinase medium [1% citrus pectin, 0.14% $NH_4 (SO_4)_2$, 0.6% KH_2PO_4, 0.2% K_2HPO_4 and 0.01% $MgSO_4.7H_2O$; pH 6.0] and incubated at 30°C for 48 h. The culture suspensions were centrifuged at 10,000 rpm for 10 min at room temperature.

Pectinase activity was measured colorimetrically using DNSA reagent (Miller, 1959) as follows.

0.5mL of cell free supernatant was incubated with 0.5mL of 1% pectin in 0.1 M acetate buffer (pH 6.0) (Appendix D.1) and the reaction mixture was incubated at 40°C for 10 min in static condition. After adding 1mL of DNS reagent, the mixture was boiled for 5 min at 90°C in a water bath. The reaction was stopped by adding 1mL of Rochelle's salt. Then the mixture was diluted by adding 2mL of de-ionized water. The absorbance was measured spectrophotometrically at 595 nm. This absorbance was translated by plotting against standard curve to get μmol of glucose. A standard curve was prepared with various concentrations of glucose (10, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 μg mL^{-1}) and used in the assay. One unit of pectinase activity was defined as the amount of enzyme which liberated 1μM glucose per min.

3.2.5.5. Screening for cellulase production

3.2.5.5.1. Qualitative assay: Congo red agar (KH_2PO_4, 0.5g L^{-1}; CMC, 2g L^{-1}; MgSO4, 0.25g L^{-1}; congo red, 0.2g L^{-1}; gelatin, 2g L^{-1}; agar, 15g L^{-1}) was used to test the cellulase activity (Sazci *et al.*, 1986). Wells of 7 mm diameter were bored using a sterile cork borer. *R. solanacearum* isolates were grown in cellulase broth [Carboxymethylcellulose, 10g L^{-1}; $NH_4(SO_4)_2$, 5g L^{-1}; KH_2PO_4, 1g L^{-1}; K_2HPO_4, 1.145g L^{-1}; $MgSO_4.7H_2O$, 0.4g L^{-1}; $CaCl_2.2H_2O$, 0.05g L^{-1} and $FeSO_4.7H_2O$, 0.00125g L^{-1}; pH 7.0] and incubated at 37°C for 48 h. After 48 h of incubation, culture suspensions were centrifuged at 10,000 rpm for

12 min and the supernatant was collected and filtered through 0.45 µm filter to be used in the assay medium. Standard concentrations (5.0, 0.5, 0.05, 0.005 and 0.0005 mg mL^{-1}) of cellulase were prepared using lyophized cellulase enzyme powder from *A. niger*. Two sets of Congo red agar plates were used for the assay. 40µL of cellulase standard solutions and the culture supernatants were added in the wells and plates were incubated at 28°C for 48 h. The 1st set of plates was flooded with 1% CTAB for 30 to 40 min and the 2nd set was flooded with Gram's iodine (2g KI and 1g iodine in 300 mL distilled water) for 3 to 5 min. All the plates were observed for zones of degradation. These plate assays were conducted in two replications.

3.2.5.5.2. Quantitative assay of crude cellulase (Denison and Kochn, 1977)

400µL of *R. solanacearum* isolates (8 log CFU mL^{-1}) was inoculated in 20mL of cellulase broth medium and were incubated at 37°C for 48 h. The culture suspensions were centrifuged at 10,000 rpm for 10 min at room temperature.

Cellulase activity was measured colorimetrically using DNSA reagent (Miller, 1959) as follows. 1mL of 1% CMC solution and 0.1mL of cell free supernatant were pipetted out and the mixture was incubated at 55°C for 15 min. 0.5mL DNS reagent was added after removing the mixture from water bath. This was again heated in boiling water bath for 5 min. While the tubes were warm, 1mL of 40% potassium sodium tartarate solution was added. The solution was kept for cooling at room temperature. Distilled water was added to make up the volume to 5mL and measured at A_{540} nm. This absorbance was translated to get µmol of glucose by plotting against standard curve. A standard curve was prepared with various concentrations of glucose (10, 25, 50, 100, 200, 300, 400, 500, 600, 700,

800, 900 and 1000 μg mL^{-1}) and used in the assay. One unit of cellulase activity was defined as the amount of enzyme which liberated 1μM glucose per min.

The glucose standard curve was prepared for pectinase and cellulase, the extinctions were plotted against their concentrations and thus regression equation was established using $y = mx + c$. Standard factor was calculated for glucose standard. Enzyme activity was calculated for both the enzymes using the formula (Absorbance of the test x standard factor/time of incubation) and eventually the enzyme activity in U was derived for both the enzymes.

3.2.5.6. Isolation and characterization of EPS

R. solanacearum isolate Rs-08-17 was used for EPS production and characterization. It was grown in CPG broth for 18 to 24 h.

3.2.5.6.1. Alcian blue staining of bacterial EPS

Alcian blue staining of EPS produced by Rs-08-17 was performed to investigate its acidic or basic nature. Alcian blue is cationic dye used to stain acidic polysaccharides. *R. solanacearum* isolate grown in CPG medium was smeared on a glass slide and air dried. The slide was flooded with 20μL of 0.1% Alcian blue dye in acetic acid (pH 2.5) for 5 min. Destaining was done gently in running water followed by air drying and observation under oil immersion lens of light microscope (Alldredge *et al*., 1993; Bhaskar and Bhosle, 2005).

3.2.5.6.2. EPS production, extraction and dialysis

R. solanacearum isolate was grown to mid-log phase (OD_{600} of 1.1 to 1.5) in CPG broth. EPS was precipitated by adding 4 volumes of acetone at 4°C overnight. Simultaneously, dialysis tubing was washed with running water for 3 to 4 h (to remove glycerine). Later

the dialysis tube was treated with 0.2% Na_2SO_3 at 80°C for 1 min followed by washing with water at 60°C for 2 min (to remove Na_2SO_3). Again it was acidified with 0.25% solution of H_2SO_4 for 5 min and rinsed with hot water (to remove acid). Finally it was washed with cold water and soaked in distilled water (stored at 4°C). The extracted crude EPS was dialyzed extensively against double distilled water in the treated dialysis tubing, lyophilized, and then stored at -20°C until further analysis.

3.2.5.6.3. Fourier-transformed infrared (FTIR) spectroscopic analysis of bacterial EPS

To detect functional groups present in the EPS, FTIR was carried out by micro-KBr pellet technique (Bramhachari *et al*., 2007). 2mg of lyophilized EPS powder was ground with 200mg of dry potassium bromide and fine pellet of EPS was prepared. This pellet was pressed into a 16 mm diameter mold and a spectrum was recorded using Shimadzu FTIR, model 8201PC (Shimadzu, Japan) in the frequency range of 400-4000 cm^{-1}.

3.3. Results and Discussion

3.3.2. Virulence testing of *R. solanacearum* isolates on eggplant

Virulence of all the 50 isolates was tested on 25-day old eggplant seedlings by inoculating a defined inoculum of cells (7 log CFU ml^{-1}) by using two methods viz. soil drenching and petiole inoculation (Fig. 3.1). Disease severity index was assessed at weekly intervals until 30 days post inoculation.

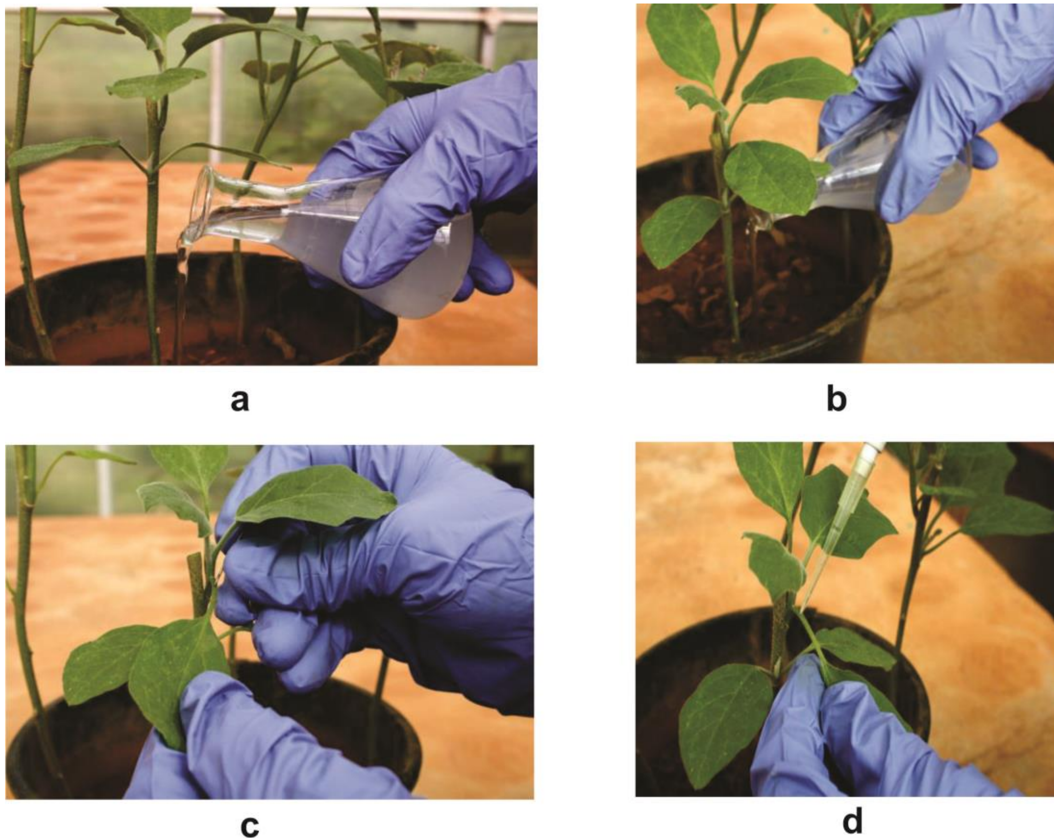


Fig. 3.1 Virulence testing on eggplant for determining the pathogenicity of *R. solanacearum* isolates by (a, b) soil drenching method i.e. pouring the inoculum in the rhizosphere region and by petiole inoculation i.e. puncturing the stem using needle (c) and inoculating 2 μL of the pathogen suspension (d).

Results of pathogenicity using soil drenching method indicated that 2 isolates (Rs-08-17 and Rs-10-337) wilted 100% eggplant within 7 days post inoculation, thus proved to be highly virulent isolates amongst all the other isolates under study. However, 7 isolates

viz. Rs-08-61, Rs-09-104, Rs-09-153, Rs-09-189, Rs-10-216, Rs-10-277 and Rs-10-298 failed to cause the infection in the host (Table 3.1). Sixteen isolates caused wilt in the range of 26-50% and 11 isolates caused wilt in the range of 51-75% post 7 days of inoculation; 13 isolates wilted 51-75% plants and 12 isolates wilted 76-100% plants after 15 days of inoculation by this inoculation method. *R. solanacearum* generally invades its host through wounds in the roots and spreads rapidly through the plant. The typical wilting symptoms result from an excessive production of extracellular polysaccharides (EPS) within the vascular system, thus altering water fluxes in the plant (Genin *et al.*, 2005).

Results of pathogenicity using petiole inoculation method indicated that 7 isolates viz. Rs-08-43, Rs-08-44, Rs-10-209, Rs-10-325, Rs-10-337, Rs-11-341 and Rs-11-357 caused 100% wilting within 7 days post inoculation. However, 3 isolates viz. Rs-09-153, Rs-09-189 and Rs-10-216 failed to cause the infection in the host till the last day of observation after pathogen inoculation (Table 3.1). Higher number of plants (76-100%) wilted post 14 days of inoculation. 4 isolates viz. Rs-08-61, Rs-09-104, Rs-10-277 and Rs-10-298 did not cause wilting when inoculated by soil drenching but wilted the plants when inoculated through petiole. All the isolates showed disease incidence in the range of 10-100% by both the methods. Jaunet and Wang (1999) determined the pathogenic variation among the isolates present in tomato fields and hypothesised that the variability that was observed in *R. solanacearum* population can also be related to its soilborne nature. From literature, it is known that the most virulent and aggressive phylotype I strains belonging to biovar 3, 4, and 5 have only been reported in Asia (Truong *et al.*, 2008).

Table 3.1 Percent wilt and Percent disease severity index (PSI) in eggplant inoculated with *R. solanacearum* by soil drenching and petiole inoculation methods

No.	Isolate	Percent Disease (%)											
		7 DAI			14 DAI			21 DAI			28 DAI		
		SD	PSI	PI	SD	PSI	PI	SD	PSI	PI	SD	PSI	PI
1	Rs-08-01	50±5	40±2.5	80±0	70±5	75±2.5	80±5	70±5	75±2.5	80±0	70±5	75±0	80±5
2	Rs-08-17	100±0	97.5±15	100±0	100±0	100±10	100±5	100±0	100±5	100±5	100±0	100±15	100±0
3	Rs-08-43	70±0	70±5	100±0	100±5	100±10	100±5	100±5	100±2.5	100±5	100±5	100	100±0
4	Rs-08-44	60±0	60±10	100±5	90±5	90±10	100±0	90±5	90±2.5	100±0	90±5	90±0	100±0
5	Rs-08-47	80±5	80±10	90±0	90±5	90±15	100±0	90±5	90±15	100±5	90±5	90±0	100±0
6	Rs-08-49	30±0	25±5	90±5	70±0	70±2.5	100±5	70±0	70±15	100±0	70±0	70±0	100±0
7	Rs-08-61	0	0	80±5	0	0	90±5	0	0	100±5	0	0	100±0
8	Rs-08-74	40±5	5±2.5	10±0	40±0	10±10	10±5	40±0	10±2.5	10±0	40±0	10±15	10±5
9	Rs-09-104	0	0	40±0	0	0	40±0	0	0	40±0	0	0	40±5
10	Rs-09-113	0	0	20±0	20±0	20±2.5	70±5	40±0	37.5±5	70±0	40±0	40±15	70±5
11	Rs-09-123	0	0	0	40±0	27.5±10	20±5	40±5	40±2.5	30±5	40±5	50±0	30±0
12	Rs-09-153	0	0	0	0	0	0	0	0	0	0	0	0
13	Rs-09-157	60±0	32.5±5	70±5	70±0	70±5	100±0	70±0	70±2.5	100±0	70±0	70±0	100±0
14	Rs-09-172	0	0	20±0	20±0	15±15	20±5	20±0	20±5	20±0	20±0	20±0	20±5
15	Rs-09-173	10±0	5±0	30±5	30±5	22.5±5	30±0	30±5	30±15	30±0	30±5	30±15	30±5
16	Rs-09-176	0	0	40±5	40±5	25±10	40±5	40±5	40±5	40±5	40±5	40±0	40±5
17	Rs-09-189	0	0	0	0	0	0	0	0	0	0	0	0
18	Rs-09-197	0	0	0	20±5	20±2.5	100	20±5	20±0	100±5	20±5	20±15	100±0
19	Rs-09-83	40±5	30±10	40±0	60±0	60±15	80±5	60±0	60±15	80±0	60±0	60±0	80±5
20	Rs-09-86	10±5	7.5±0	40±5	30±0	30±15	100±0	40±0	40±5	100±5	40±0	40±0	100±0
21	Rs-10-201	70±5	70±±5	50±0	70±5	70±7.5	50±0	70±5	70±2.5	50±0	70±5	70±0	50±5
22	Rs-10-209	50±5	32.5±0	100±5	90±5	90±2.5	100±5	90±5	90±0	100±5	90±5	90±10	100±0
23	Rs-10-216	0	0	0	0	0	0	0	0	0	0	0	0
24	Rs-10-250	30±5	7.5±7.5	40±0	40±0	32.5±10	40±5	40±0	40±2.5	40±0	40±0	40±0	40±5
25	Rs-10-262	10±0	10±5	90±5	90±0	90±7.5	100±0	90±0	100±15	100±5	90±0	100±0	100±0
26	Rs-10-266	30±5	40±7.5	60±5	50±0	42.5±2.5	70±5	50±0	50±5	80±5	50±0	50±15	90±5
27	Rs-10-268	30±5	30±0	70±5	60±0	47.5±15	50±0	60±0	60±0	70±0	60±0	60±0	85±5

No.	Isolate	Percent Disease (%)											
		7DAI			14DAI			21DAI			28DAI		
		SD	PSI	PI	SD	PSI	PI	SD	PSI	PI	SD	PSI	PI
28	Rs-10-273	40±0	50±5	80±5	60±0	60±7.5	100±5	60±0	60±0	100±5	60±0	60±0	100±0
29	Rs-10-277	0	0	80±0	0	0	90±0	0	0	90±5	0	0	90±5
30	Rs-10-278	20±0	20±10	40±5	30±5	30±10	50±5	30±5	30±5	50±0	30±5	30±0	50±5
31	Rs-10-280	10±0	20±5	40±5	30±5	30±7.5	50±0	30±5	30±0	50±5	30±5	30±10	55±5
32	Rs-10-286	20±0	20±0	80±5	40±0	40±10	90±5	40±0	40±2.5	90±0	40±0	40±0	90±5
33	Rs-10-295	30±0	25±5	90±5	50±5	50±2.5	100±5	50±5	50±5	100±5	50±5	50±5	100±0
34	Rs-10-298	0	0	0	0	0	60±0	0	0	70±0	0	0	75±0
35	Rs-10-315	70±0	70±7.5	50±5	70±5	70±10	50±0	70±5	70±15	50±0	70±5	70±0	55±0
36	Rs-10-319	70±5	70±5	40±0	70±5	70±15	50±5	70±5	70±5	50±5	70±5	70±0	50±5
37	Rs-10-322	40±5	20±0	50±0	50±5	50±2.5	50±5	50±5	50±0	60±0	50±5	50±15	70±0
38	Rs-10-325	60±5	47.5±7.5	100±5	100±0	90±7.5	100±0	100±0	100±0	100±0	100±0	100±0	100±0
39	Rs-10-332	30±0	25±0	90±5	50±0	50±2.5	100±5	50±0	50±5	100±5	50±0	50±0	100±0
40	Rs-10-337	70±0	50±0	60±5	90±0	80±10	90±0	90±0	90±2.5	90±0	90±0	90±10	90±5
41	Rs-10-337	100±0	97.5±15	100±0	100±0	100±7.5	100±5	100±0	100±15	100±0	100±0	100	100±0
42	Rs-11-340	0	0	0	30±0	27.5±5	60±0	30±0	30±15	70±5	30±0	30±0	70±5
43	Rs-11-341	60±0	60±2.5	100±5	90±5	90±15	100±5	90±0	90±5	100±0	90±0	90±15	100±0
44	Rs-08-344	0	0	80±0	0	0	80±0	20±0	0	80±5	30±5	20±5	80±0
45	Rs-11-357	70±0	60±0	100±5	100±0	97.5±2.5	100±0	100±0	100±15	100±0	100±0	100±0	100±0
46	Rs-11-359	10±0	10±7.5	90±5	90±0	90±5	100±5	90±5	100±2.5	100±0	90±5	100±5	100±0
47	Rs-11-363	70±5	70±5	30±0	70±5	70±10	50±0	70±5	70±5	50±5	70±5	70±0	50±5
48	Rs-11-364	50±0	50±0	80±5	60±0	60±2.5	100±0	60±5	60±15	100±0	60±5	60±5	100±0
49	Rs-11-365	0	0	60±0	0	0	90±5	0	0	90±5	30±0	20±0	90±5
50	Rs-11-376	40±0	30±2.5	70±5	60±0	47.5±15	70±5	60±0	60±15	70±0	60±0	60±5	70±5

SD- Soil drenching method; PI-Petiole inoculation method; PSI –Percent severity index (Calculated using the 0-4 disease index scale); Initial inoculum: 7 log CFU mL^{-1}; Number of plants: 5; ± Standard error of mean values of two replications

When both the methods were compared, it was observed that wilt incidence was observed faster in case of petiole inoculation as compared to soil drenching method. This is because the bacteria gets direct entry into the host in petiole inoculation whereas, in soil drenching inoculation method the pathogen has to break the host barriers to get inside and later has to move through the root cortex and penetrate into the xylem vessels which consumes sometime. 3 isolates viz. Rs-08-61, Rs-10-104 and Rs-10-298 caused wilting in eggplant by petiole inoculation but not when inoculated by soil drenching method. Therefore, it's likely that wilting was observed faster in case of petiole inoculation which allowed the pathogen to colonize faster into the xylem tissues as compared to soil drenching inoculation which is more natural a process to establish infection. These reports are in conformity with the findings of Saile *et al.* (1997) who also used petiole inoculation method and further reported that EPS I have an important function in facilitating the bacterial multiplication in tomato plant stems.

Maximum number of isolates (n=21) wilted 76-100% plants in petiole inoculation as compared to soil drenching where only 3 isolates caused wilt on 76-100% plants in 7 days post inoculation. Even after 14 days of inoculation, 28 isolates wilted maximum number of plants (76-100%) by petiole inoculation method as compared to soil drenching (12 isolates) (Table 3.2; Fig. 3.2). 9 isolates caused wilting in 1-25% plants within 7 days of inoculation, whereas, in the rest of the days only 2-3 isolates managed to wilt these number of plants by soil drenching method. This observation was also recorded in case of petiole inoculation (2-3 isolates caused wilting in 1-25% plants). The reason behind this high degree of variation in pathogenicity might be due to recombination process which takes place in bacteria. Similar explanations were also given by many other researchers

(Didelot and Maiden, 2010). Bertolla *et al.* (1999) also reported that *R. solanacearum* is capable of entering into the competent state in planta and is subject to horizontal gene transfer both *in vitro* (Guidot *et al.*, 2009) and in competition experiments (Coupat-Goutaland *et al.*, 2011).

Table 3.2 Comparision between number of strains causing Wilt incidence on eggplant by soil drenching (SD) and Petiole inoculation (PI) methods

Percent disease	7DAI		14DAI		21DAI		28 DAI	
	SD	PI	SD	PI	SD	PI	SD	PI
0%	15	8	9	4	8	4	7	4
1-25%	7	3	3	3	3	2	2	2
26-50%	14	12	14	11	15	11	17	9
51-75%	11	6	12	4	12	5	12	6
76-100%	3	21	12	28	12	28	12	29

SD-Soil drenching; PI-Petiole inoculation; DAI-Days after inoculation

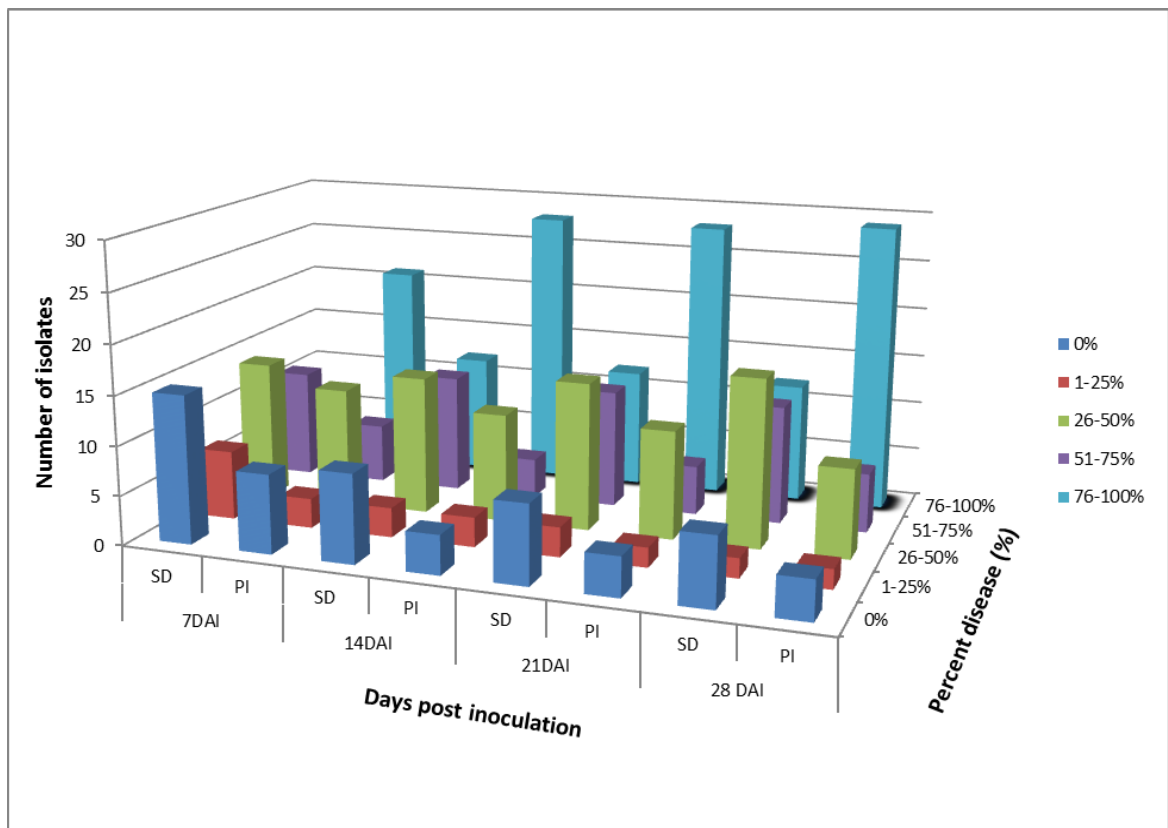


Fig. 3.2 Wilt incidence on eggplant by soil drenching (SD) and Petiole inoculation (PI) methods over a period of 28 days; Initial inoculum: 7 log CFU mL^{-1}; DAI: Days after inoculation; SD: Soil Drenching; PI: Petiole inoculation; Number of inoculated Plants: 10 in two replications.

Incidence of wilt was also recorded as percent severity index (PSI). Maximum PSI (76-100%) was exhibited by 3 isolates (Rs-08-17, Rs-08-47 and Rs-10-337) post 7 days of pathogen inoculation (Table 3.1). PSI data showed that more number of isolates (14) caused wilt in the range of 26-50% (Fig. 3.3) after 21 days post inoculation. At the end of 28 days of inoculation, 13 isolates displayed higher PSI in the range of 76-100%.

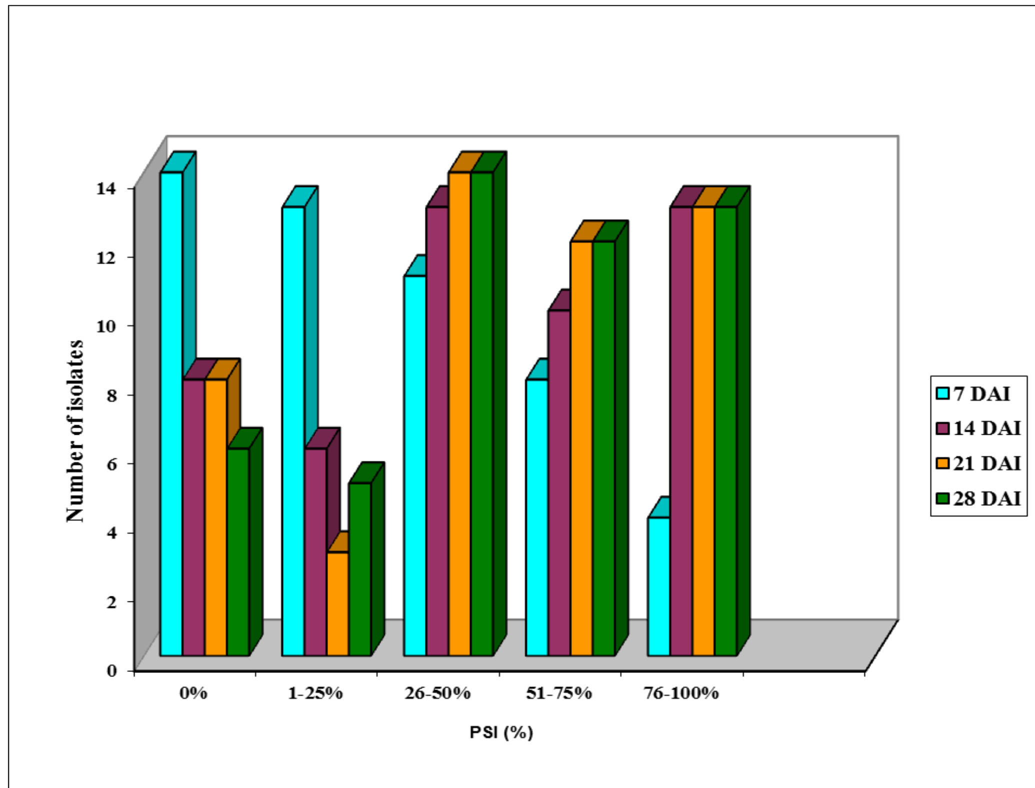


Fig. 3.3 Percent severity index (PSI) of BW in eggplant by soil drenching method over a period of 28 days. PSI was derived on 0-4 disease scale (Calculated based on formula given by Cooke, 2006); DAI- Days after inoculation; Number of inoculated Plants: 10 in two replications.

3.3.3. Pathogenicity of non-EPS *R. solanacearum* isolate on eggplant

To investigate whether non-eps producing isolate could also cause severe wilt in the eggplant, the pathogenicity studies were conducted with *R. solanacearum* isolate Rs-08-17 (wild type) and Rs-100C (EPS mutant) on susceptible cultivar *Agassiam* by means of soil drenching inoculation. Results indicated that only Rs-08-17 was pathogenic on

eggplant, whereas, Rs-100C failed to infect the eggplant host (even at a higher concentration of inoculum (9 log CFU mL^{-1}). There was no ooze detected in the eggplant inoculated with Rs-100C. It was also observed that as the inoculum population increased, the rate of wilt incidence also enhanced when Rs-08-17 was inoculated. More number of plants wilted at a higher cell density as the Rs-08-17 population increased (Table 3.3). The single most important virulence factor of *R. solanacearum* is its high molecular mass EPS, which is produced in massive amounts in culture and in planta (Denny, 2006). All virulent wild-type strains of *R. solanacearum* produce EPS (Husain and Kelman, 1958; Buddenhagen and Kelman, 1964; Poussier *et al.*, 2003), while EPS-deficient mutants are avirulent.

Table 3.3 Percent bacterial wilt in eggplant inoculated with wild type *R. solanacearum* Rs-08-17 and a non EPS producing Rs-100C isolate

		Wilt incidence (%)						
Inoculum density	**Isolate**	7DAI	11DAI	13DAI	15DAI	21 DAI	25DAI	28DAI
7 log CFU mL^{-1}	Rs-08-17	60±5	60±5	90±0	100±0	100±5	100±0	100±5
	Rs-100C	0	0	0	0	0	0	0
8 log CFU mL^{-1}	Rs-08-17	80±0	90±0	100±5	100±0	100±0	100±0	100±0
	Rs-100C	0	0	0	0	0	0	0
9 log CFU mL^{-1}	Rs-08-17	90±0	90±5	100±5	100±0	100±0	100±0	100±0
	Rs-100C	0	0	0	0	0	0	0

Isolates: Rs-08-17 (highly virulent) and Rs-100C (non-EPS producer)
DAI- Days after inoculation; Experiment was repeated with two replications
± Standard error of mean values of two replications

It has been reported earlier that EPS mutants fail to cause wilt symptoms even when introduced directly into stem wounds although they remain slightly pathogenic (Denny and Baek, 1991). Production of EPS could therefore be the major reason for the *R. solanacearum* isolate to cause severe wilting once it enters the host, whereas, an EPS mutant fails to do so as it cannot obstruct the xylem vessel fluid movement in the plant

(Denny, 1995). However, it has been reported that EPS mutants can multiply more slowly, and colonize poorly the stem of plants in systemic colonization of tomato plants when inoculated via unwounded roots (Saile *et al*., 1997; Araud-Razou *et al.*, 1998). But, the reports of this study are in congruent with the findings as reported by Denny (1995).

3.3.4. Histopathology studies of eggplant infected with bacterial wilt

Cross sections of wilted eggplant stem were observed for finding changes inside the tissue. This was compared with the healthy eggplant stem. Large sized cortical cells were observed in the stem. These were loosely packed with big intercellular spaces (Fig. 3.4 a). Necrosis of xylem (Fig. 3.4 b, c) tissue was seen in and around the cells with brown pigmentation. These observations suggest that *R. solanacearum* may enter easily through the loosely arranged cortical cells from the soil and disintegrates the xylem, phloem vessels and the connected tissues which lead to releasing of phenolic compounds. These later oxidize to quinines, giving a black discoloration in the xylem and phloem vessels of *Agassiam* variety.

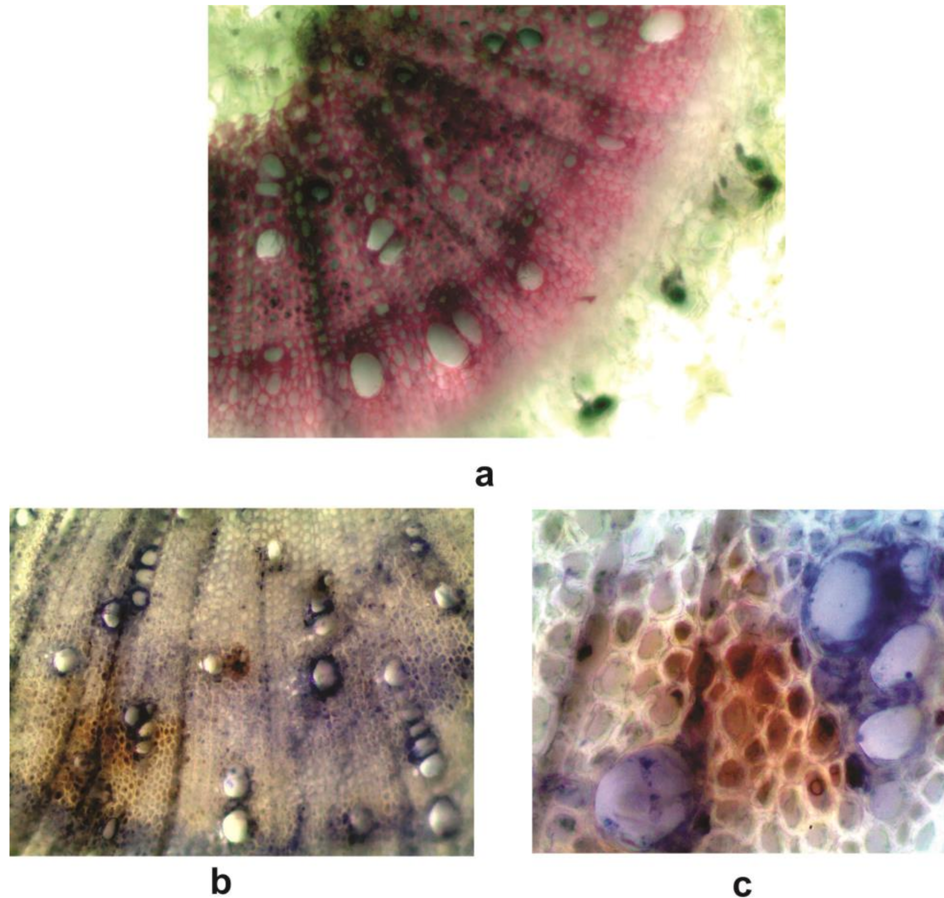


Fig. 3.4 Histopathology of eggplant stem: Cross sections of a (a) healthy eggplant stem; (b) BW infected eggplant stem showing vascular browning in between medullary rays under 40X magnification and (c) Necrotic portions observed in the xylem of eggplant stem tissue as a result of BW infection post 7 days observed under 100X magnification.

A detailed study was conducted by Wallis and Truter (1978) on the histopathology of tomato plants infected with *R. solanacearum* to determine the disease spread and destruction of vascular tissue. Researchers have revealed the association of bacterial pathogens with the presence of tyloses in xylem and of vascular tissues browning in the vicinity of the invaded xylem vessels (Beckman, 1987). Initially the vascular ring appears yellow and later turns brown (Champoiseau *et al.*, 2009). Plants accumulate different phenolic compounds in the xylem in response to infection (Baidez *et al.*, 2007) and these phenolic compounds could also activate other defense responses against vascular wilt

pathogens. Mace *et al.* (1963) have earlier confirmed that oxidized phenols rapidly undergo polymerization and empty its contents into xylem vessel. Also callose deposition and swelling of the primary walls of the xylem vessels was reported during the interaction of *R. solanacearum* with chilli pepper (Rahman *et al.*, 1999).

3.3.5. Study on the virulence factors of *R. solanacearum*

3.3.5.1. *R. solanacearum* isolates

Five isolates viz. Rs-08-17 (highly pathogenic), Rs-09-100 (moderately pathogenic), Rs-09-109 (weakly pathogenic), Rs-100C and Rs-100D (non-eps producing colonies of *R. solanacearum*) were selected based on the pathogenic characteristics and EPS production for studying the major virulence factors.

3.3.5.2. Motility and chemotaxis Assays

3.3.5.2.1. Motility of *R. solanacearum*

In order to visualize the bacterial motility, phase contrast microscopy was used. It was observed that Rs-08-17, Rs-09-100 and Rs-09-109 cells moved very rapidly from the center to the edge of the coverslip and were therefore regarded to be highly motile (Fig. 3.5 a). Whereas, Rs-100C and Rs-100D cells seemed to be less motile compared to other strains exhibiting only Brownian movement. All the isolates of *R. solanacearum* displayed swimming motility on 0.3% agar plates within 24 h of incubation (Fig. 3.5 b). Smarming motility was observed only on BG agar plates with 0.5% agar after 24-48 h (Fig 3.5 d, e, f). A typical bull's eye formation along with the flare type of smarming pattern was observed in the pathogen swarm colonies. The diameter of 9 mm was recorded for Rs-08-17, Rs-09-100 and Rs-09-109 isolates (Table 3.4). Rs-100C and Rs-

100D produced a diameter of 8 mm, whereas, when the isolates were inoculated on the BMM medium with 0.4% glucose no swarming motility was observed.

It has been reported earlier by Tans Kersten *et al.* (2001) that *R. solanacearum* needs motility for full virulence, apparently because motility facilitates the early stages of host plant invasion and colonization. It has been shown that swimming motility in *R. solanacearum* contributes largely as the pathogen moves towards the roots of host plants (Mao and He, 1998), proliferates rapidly the intercellular spaces of the root cortex, followed by colonization of the intercellular spaces in the inner cortex and the vascular parenchyma. All the isolates under study exhibited the swimming and swarming motility. Swarming motility is powered by rotating flagella as a result of which the bacteria moves rapidly (Henrichsen, 1972).

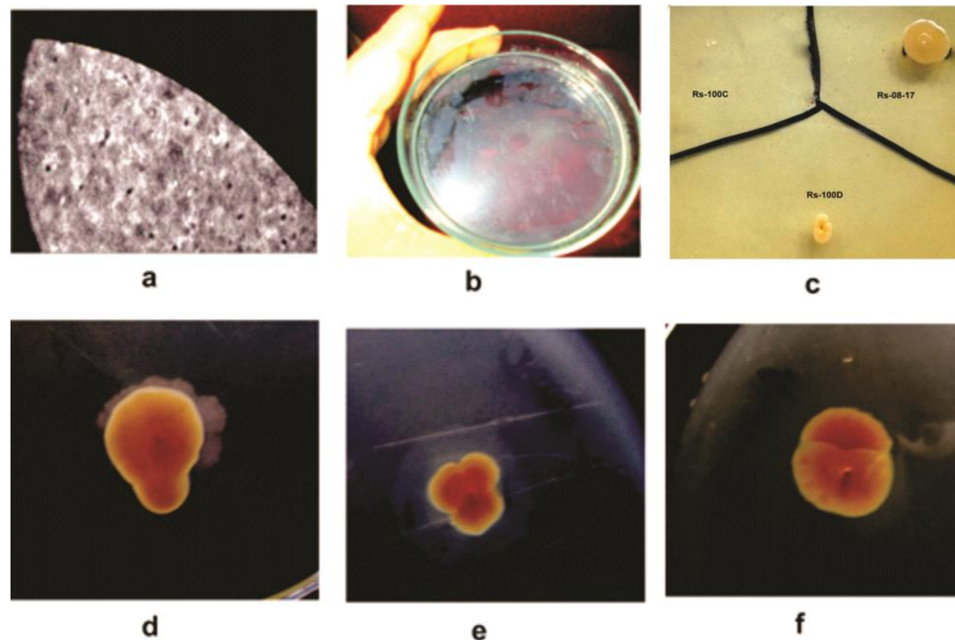


Fig. 3.5 Photo images of *R. solanacearum i*solate (a) Motility by hanging wet drop method as observed under phase contrast microscopy*;* (b) Swimming motility and (c) Twitching motility displayed by Rs-08-17; Swarming motility on BG agar exhibited by (d) Rs-08-17, (e) Rs-100D and (f) Rs-100C isolates.

Table 3.4 Motility assays of isolates with specific swimming, swarming and twitching phenotypes and siderophore and EPS producers

Isolates	**Motility assays**				**Siderophore production**	**EPS production**
	Swimming[a]	Swarming[b]		Twitching[c]	Zone size (mm)	
		BMM	BG agar zone (mm)			
Rs-08-17	+	-	9	+	20	+
Rs-09-100	+	-	9	+	18	+
Rs-09-109	+	-	9	+	18	+
Rs-100C	+	-	8	-	15	-
Rs-100D	+	-	8	-	14	-

Wild type isolates: Rs-09-17, Rs-09-100 and Rs-09-109; Non EPS producers: Rs-100C and Rs 100D ; + indicates presence; - indicates absence; [a]Individual movement in liquid powered by rotating flagella; [b]Multicellular surface movement powered by rotating helical flagella; [c]surface movement powered by the extension and retraction of pili

R. solanacearum isolates Rs-08-17, Rs-09-109 and Rs-09-100 exhibited twitching motility on BG agar plates, layered edges with multiple irregular concentric circles of growth emerging out were observed on the plates which further grew and formed a wavy pattern on the agar surface (Fig. 3.5 c). No twitching motility was observed in Rs-100C and Rs-100D. *R. solanacearum* uses twitching motility to move within the host (Liu *et al.*, 2001). This motility is driven by retractable type 4 pili (Mattick, 2002). It's required for bacterial adherence to host cells and natural transformation (Kang *et al.*, 2002). Type IV pili mutants of *R. solanacearum* strains were less virulent (Liu *et al*., 2001; Kang *et al*., 2002). This explains the significant role played by twitching motility in *R. solanacearum* pathogenesis. The type IV pili formation might be contributing to bacterial pathogenesis by invading the host and colonization of cortex resulting in BW. The results indicate that since this motility was observed only in case of Rs-08-17, Rs-09-100 and Rs-09-109 and not in mutants, pilus formation probably contributes to pathogenesis during both invasion of roots and inside the plant by promoting adherence to host cell surfaces, colonization of root surfaces, migration to wound sites. This may lead to biofilm

formation in the plant on host xylem vessel walls. This would further help the pathogen from host defenses and help bacterial survival during latent infections (Genin and Boucher, 2004).

3.3.5.2.2. Chemotaxis

Eggplant seedlings were grown on water agar plates (Fig. 3.6 a) for collection of root exudates. Chemotactic response was indicated by presence of halo produced by Rs-08-17 motile cells around the root exudate inoculation site (Fig 3.6 b), no such behavior was observed in case of mutant strains. *R. solanacearum* Rs-08-17 was tested for its growth on sole carbon sources to identify the chemicals that trigger the chemotaxis response. Rs-08-17 showed a strong chemotactic response to all organic acids tested, except succinic acid, but was not attracted to alanine, glycine among the amino acids (Table 3.5). Also it showed strong attraction towards glucose and galactose, but failed to grow in presence of sucrose, rhamnose and fructose. Whereas, the other two mutant strains were attracted towards glucose, galactose, saccharose, galacturonic acid and aspartate only.

Motility in coordinated way is essential for full virulence rather than random motion (Yao and Allen, 2006). This was triggered by the presence of root exudates thus allowing the pathogen to behave and exhibit chemotactic response (Yao and Allen, 2006).

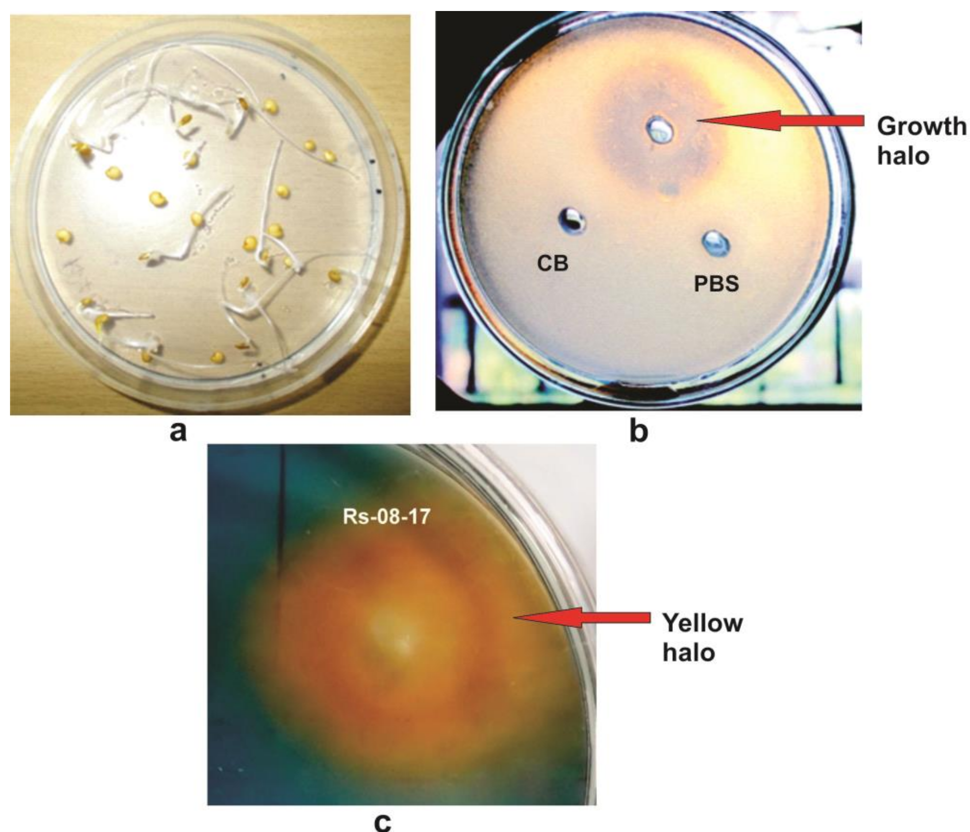


Fig. 3.6 (a) Germination of eggplant seeds on water agar plates for collection of root exudates, (b) a strong positive chemotactic response shown by *R. solanacearum* Rs-08-17 isolate towards eggplant root exudates, (c) Siderophore production as indicated by a distinct yellow halo.
CB-Chemotaxis buffer, PBS-Phosphate saline buffer

Table 3.5 Chemotactic response of wild type and mutant *R. solanacearum* isolates to sugars, organic acids and amino acids

No.	**Compounds**	**Carbon sources**	**Utilization***		
			Rs-08-17	Rs-100C	Rs-100D
1	Sugars (10 mM)	Glucose	++	++	++
		Fructose	-	-	-
		Galactose	++	+	+
		Saccharose	+	++	++
		Trehalose	+	-	-
		Rhamnose	-	-	-
		Sucrose	-	-	-
		Xylose	+	-	-

No.	Compounds	Carbon sources	Utilization*		
			Rs-08-17	Rs-100C	Rs-100D
2	Organic acids (10 mM)	Citric acid	++	-	-
		Malic acid	+	-	-
		Succinic acid	-	-	-
		Glucuronic acid	+	-	-
		Galacturonic acid	++	++	++
3	Amino acids (4 mM)	Alanine	-	-	-
		Glycine	-	-	-
		Tryptophane	+	-	-
		Aspartate	+	+	+

* 3 isolates were used for determining the utilization of sugars (10 mM), organic acids (10 mM) and amino acids (4 mM) as sole carbon sources in BMM broth within 24 h.
- indicates no growth; + indicates moderate growth; ++ indicates vigorous growth

Plant roots release a wide range of compounds viz. sugars and amino acids that are involved in attracting strongly and selectively many bacterial pathogens and forming the special associations in the rhizosphere (Brencic and Winans, 2005). The root-derived substrates that attract *R. solanacearum* may vary suggesting that chemotactic responses can be specific for different hosts or ecological niches (Zhulin *et al.*, 1995, Yao and Allen, 2006). This pathogen moves towards plant roots as a result of presence of amino acids, organic acids in the root exudates. It has been reported by Li *et al.* (2009) that the amount of amino acids and soluble sugar were positively and significantly correlated with the disease indices. This study can therefore infer that eggplant root exudates could possibly be one of the key reasons for the observed higher incidence of bacterial wilt in the wild type *R. solanacearum* Rs-08-17 isolate. However, when the pathogenicity testing was conducted with non-tactic mutant strain Rs-100C by soil drenching method, it failed

to cause infection. This indicated that a taxis is probably an important factor in the early stages for successful invasion of host tissues.

3.3.5.3. Siderophore Production

All the strains produced siderophores when screened on CAS agar medium by forming a halo around the colony (Fig. 3.6 c, Table 3.4). However, in this study all the isolates produced different halos; Rs-08-17 produced the largest zone (20mm). These siderophores are correlated with the virulence of many pathogenic microbes (Crichton and Charloteaux-Wauters, 1987). *R solanacearum* strain K60 had been reported to produce the dibydroxamate siderophore called schizokinen and polycarboxylate siderophore stapbyloferrin B (Bhatt and Denny, 2004). However, a study has also reported a mutant strain that does not produce staphyloferrin B yet is virulent on tomato host. It is therefore too early to conclude the role played by siderophores in pathogenesis.

3.3.5.4. Screening for Pectinase production

All the 5 isolates were tested qualitatively for pectinase production on simple pectin medium and assay medium. The zones of degradation were produced on assay plate within 17 h of incubation and on simple pectin medium by 3 days.

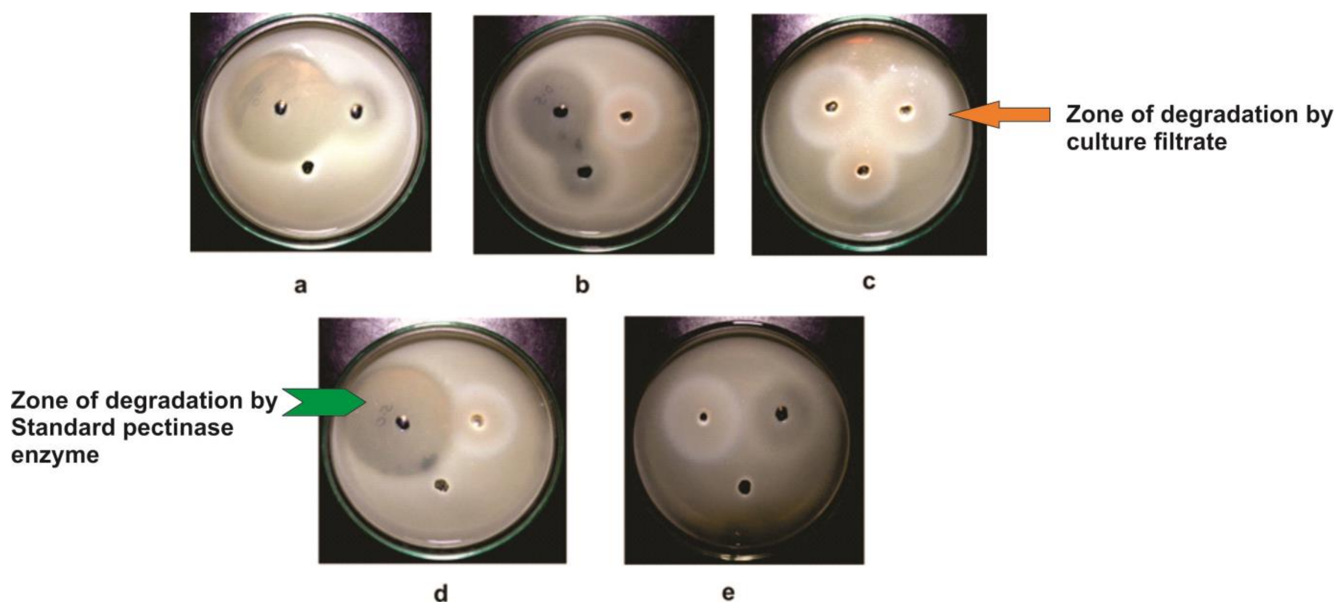


Fig. 3.7 Zones of degradation by pectinase enzyme on pectin agar plates (b, c, d, e) along with pectinase enzyme standard from *A. niger* (a, b, d).

In the pectinase plate assay, the highest activity on both the assay medium and pectin medium was exhibited by Rs-08-17 (22 mm, 35.5 mm respectively), followed by Rs-08-100 and Rs-09-109 in plate assays (Table 3.6; Fig. 3.7).

Table 3.6 Production of pectinase by *R. solanacearum* isolates and its enzymatic activity

	Qualitative Plate assay				**Pectinase colorimetric assay**			
	Assay medium		**Simple pectin**		**Glucose (μg ml^{-1})^{a}**	**Enzyme activity**		**Pectinase activity (U)**
Isolates	ZOD (mm)*	mg mL^{-1}	ZOD (mm)*	mg mL^{-1}		A[#]	B[$]	A/B
Rs-08-17	22±1.4	1.88	35.5±0.3	1.87	757.38	0.31	4.21	0.074
Rs-0-100	20±0.7	1.54	32.5±1.0	1.68	771.19	0.32	4.28	0.074
Rs-09-109	18.5±1.4	1.28	33.5±0.3	1.74	682.53	0.28	3.79	0.074
Rs-100C	0	0	0	0	666.41	0.28	3.70	0.074
Rs-100D	0	0	0	0	576.58	0.24	3.20	0.075

Enzyme standards were prepared for qualitative and quantitative assays: 5.0, 0.5, 0.05, 0.005 and 0.0005 mg mL^{-1}; [a] Calculated based on standard curve; Standard factor =22.22; [#]A595 x Standard factor/total volume in µmol mL^{-1} min^{-1} ; [$]Amount of glucose liberated/MW of glucose in U mL^{-1} min^{-1}; ZOD- Zones of degradation; *Mean of 3 replications;; ± Standard error of mean

However, no zones of degradation were produced by Rs-100C and Rs-100D isolates. The zone diameter was higher on the pectin medium as compared to the assay medium. The pectinase enzyme concentration on the plate assay was calculated in range of 1.28 to 1.88 mg mL^{-1} (Table 3.6)

A standard curve for glucose was determined with appropriate dilutions (Fig. 3.8). Quantitative experiments using dinitrosalicyclic acid reagent method showed pectinase activity of 0.074 U in all the isolates (Table. 3.6).

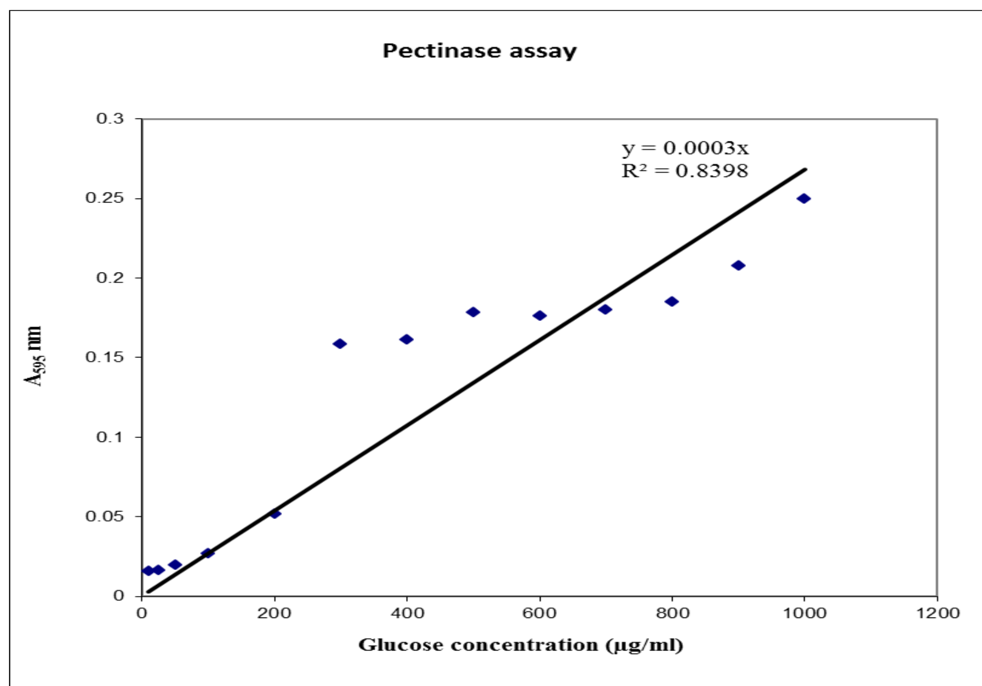


Fig. 3.8 Standard curve for reducing sugar assay from 10-1000 µg glucose mL^{-1} at A595nm by DNSA method; Regression analysis: y = 0.0003x, R^2= 0.83

3.3.5.5. Screening for cellulase production

All the 5 isolates were tested qualitatively for cellulase production on Congo red agar. The zones of degradation were produced on assay plate within 3 days of incubation. Zones of degradation of CMC were observed using 1% CTAB (Fig. 3.9 a, b, c) and Gram’s iodine solution (Fig. 3.9 d, e, f).

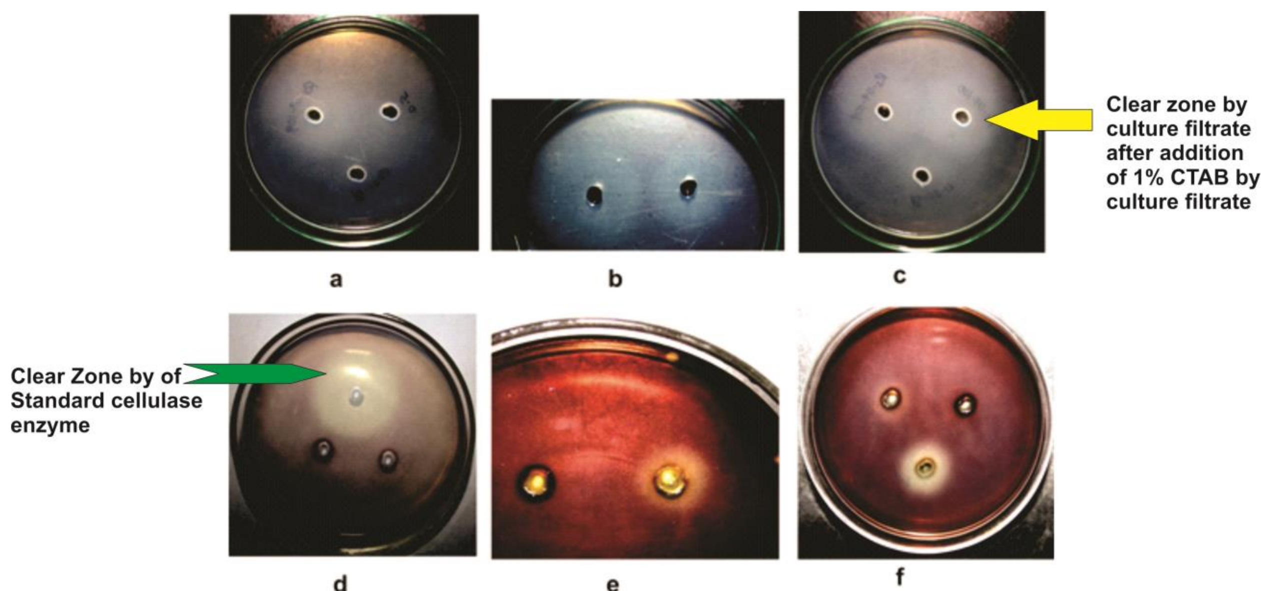


Fig. 3.9 Zones of clearance on CMC agar plates after 48 h of incubation after addition of 1% CTAB solution (a, b, c) and Gram's iodine (d, e, f) along with cellulase enzyme standard from *A. niger*. The formation of clearing zone around the culture filtrate confirms the secretion of extracellular cellulase.

In this plate assay experiment, there was a slight variation in the zones of degradation amongst the isolates (Table. 3.7).

Table 3.7 Production of cellulase by *R. solanacearum* isolates and its enzymatic activity

	Qualitative Plate assay				**Cellulase colorimetric assay**			
	1% CTAB		**Gram's iodine**		**Glucose ($\mu g\ ml^{-1}$)[a]**	**Enzyme activity**		**Cellulase activity (U)**
Isolates	ZOD (mm)*	mg mL^{-1}	ZOD (mm)*	mg mL^{-1}		A[#]	B[$]	A/B
Rs-08-17	19.5±3.5	1.5	17±1.4	1.21	589.70	0.17	3.28	0.05
Rs-0-100	22±1.8	1.8	15±2.1	0.95	590.79	0.17	3.28	0.05
Rs-09-109	20±2.8	1.56	15±1.4	0.95	747.97	0.22	4.16	0.05
Rs-100C	0	0	0	0	612.57	0.18	3.40	0.05
Rs-100D	0	0	0	0	661.94	0.19	3.68	0.05

Enzyme standards were prepared for qualitative and quantitative assays: 5.0, 0.5, 0.05, 0.005 and 0.0005 mg mL^{-1}; [a] Calculated based on standard curve; Standard factor =23.14; [#]A540 x Standard factor/total volume in µmol mL^{-1} min^{-1} ; [$]Amount of glucose liberated/MW of glucose in U mL^{-1} min^{-1}; ZOD- Zones of degradation; *Mean of 3 replications;; ± Standard error of mean

No zones of degradation were produced by Rs-100C and Rs-100D isolates. The zone diameter was higher when 1% CTAB was used as compared to Gram's iodine. The

cellulase enzyme concentration on the plate assay was calculated in range of 0.95 to 1.8 mg mL^{-1}.

A standard curve for glucose was determined with appropriate dilutions (Fig. 3.10). Quantitative experiments using dinitrosalicyclic acid reagent method showed cellulase activity of 0.05 U in all the isolates (Table. 3.7).

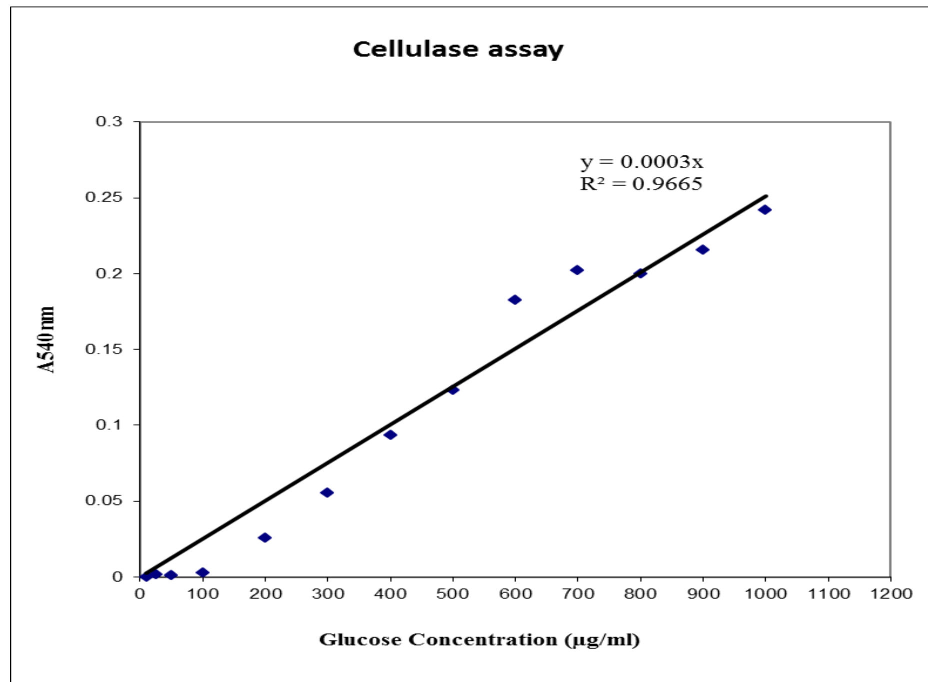


Fig. 3.10 Standard curve for reducing sugar assay from 10-1000 µg glucose/mL at A540 nm by DNSA method; Regression analysis: y = 0.0003x, R2= 0.9665

During plant infection, cell wall degrading enzymes play a very important in the colonization and multiplication in planta (Kang *et al.*, 1994). Pectin degrading enzymes are known to facilitate bacterial invasion by digesting the pectic gels formed at the secondary root emergence wounds (Vasse *et al.*, 1995). After pathogen colonization they help in degrading the pectin pit membrane and allow the host to form gels and tyloses that obstruct the water transport and result in wilting. This study has proved that the cellulase and the pectinase enzymes are produced by wild type and mutant *R.*

solanacearum isolates in colorimetric assay. However, mutant strains failed to produce zones of degradation in plate assays. Also, it was seen that not all the isolates could cause BW in eggplant when tested. Only Rs-08-17 was found to be highly virulent. Rs-100C failed to infect eggplant when tested for its pathogenic activity even at a higher inoculum density despite the fact that it could produce the enzymes. Liu *et al.* (2005) reported that GMI1000 mutants lacking one pectic enzyme (*PehA*, *PehB* or *PehC*) are slightly less virulent than GMI1000, but mutants lacking two (*PehA* and *PehB*) or three pectic enzymes (*PehA*, *PehB* or *PehC*) are at least as virulent as wild-type GMI1000.

Recently, a mutant lacking the six major plant cell wall degrading enzymes proved to be pathogenic by infecting the host slowly than the wild-type strain (Liu *et al.*, 2005). It was also shown that pectin catabolism does not significantly contribute to bacterial fitness inside the plant (Gonzalez and Allen, 2003), indicating that cellulase and pectinolytic activities are preferably required for host colonization rather than for bacterial nutrition. It is therefore unclear to what extent these enzymes contribute to the virulence of *R. solanacearum*. The results from this study prove that enzymes alone may not be essential for causing disease, each factor directly or indirectly contributes to the wild type's ability to cause wilt. The findings are congruent with the earlier work carried out by Denny *et al*. (1990) and Huang and Allen (2000).

3.3.5.6. Isolation and characterization of EPS

Rs-08-17 produced a lot of eps in the medium (Fig. 3.11a) turning off-white in colour and the EPS produced was stained using Alcian blue and observed under Olympus CX41 phase contrast microscope as extensive dense matrix and mucous mass surrounding the

cells (Fig. 3.11 b). Alcian blue staining was performed to determine its nature and since it all stained blue, it was anionic in nature.

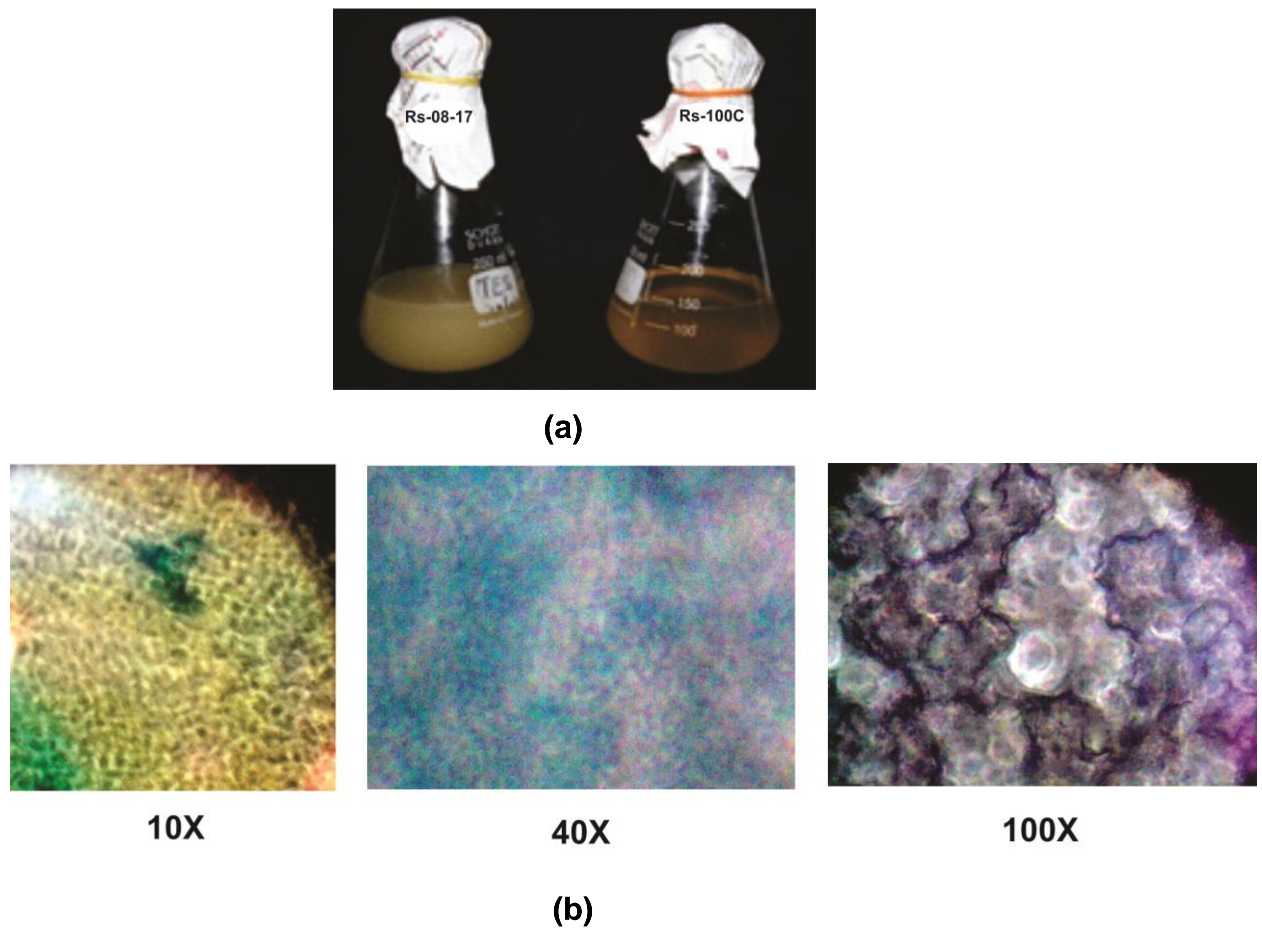


Fig. 3.11 EPS production by (a) Rs-08-17; Bright field microscopic images of EPS of *R. solanacearum* (b) Rs-08-17 stained with Alcian Blue under 10X, 40X and 100X magnification.

Alcian Blue is a stain specific to acid mucopolysaccharides (Passow and Alldredge, 1995). It confirms the fact that EPS is chiefly composed of polysaccharides. EPS1 is found to be composed of N-acetyl-galactosamine, N-acetyl-galactosaminuronic acid and bacillosamine derivative (Trigalet- Demery *et al.*, 1993).

Husain and Kelman (1958) found that production of exoploysaccharides play an important role in the pathogenicity of tomato. The results of this study have stated that Rs-08-17 produces lot of EPS and causes 100% BW. However, it has been reported that

EPS mutants can multiply more slowly and rarely wilt plants. They generally only colonize the roots and lower stems and do not spread well throughout the plant (Saile *et al*., 1997; Araud-Razou *et al.*, 1998). This could be one of the reason for the failure of Rs-100C to cause infection. Microscopic studies confirmed the accumulation of large amounts of bacterial EPS within infected vessels (Wallis and Truter, 1978), and demonstrated that a spontaneous EPS-deficient mutant strain of *R. solanacearum* was unable to invade protoxylem vessels (Vasse *et al.*, 1995).

The IR spectra of the EPS produced by Rs-08-17 indicate the major functional groups and chemical bonds present in EPS (Fig 3.12). The peaks are observed at 3510 cm^{-1} and 1409 cm^{-1} for OH and 1064 cm^{-1} for C-O (H), showed the presence of sugars. The broad peak at around 1653 cm^{-1} confirmed the presence of uronic acids in the EPS.

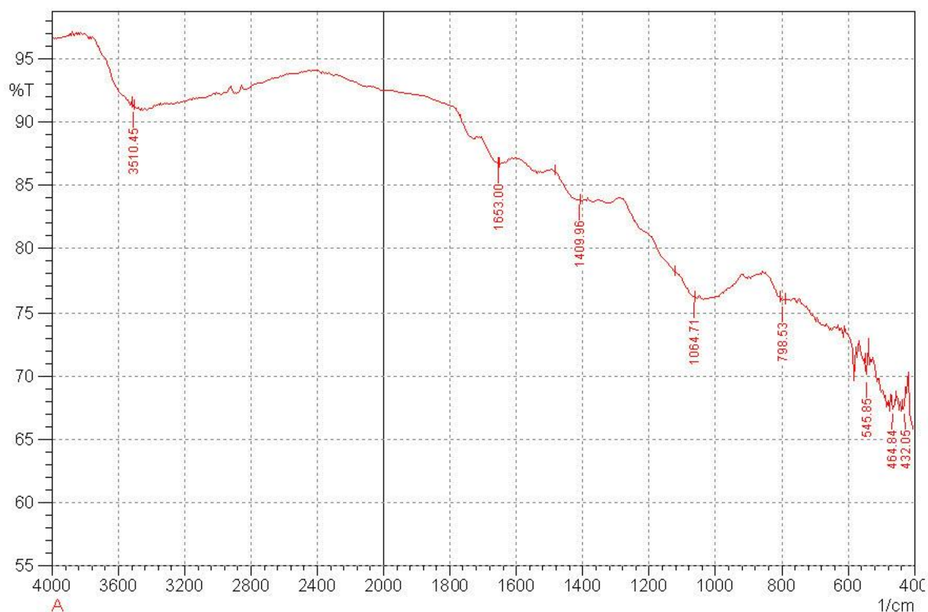


Fig. 3.12 Fourier-transformed infrared (FTIR) spectroscopic analysis of bacterial EPS produced by *R. solanacearum* Rs-08-17 isolate grown in CPG broth. A spectrum was recorded in the frequency range of 400-4000 cm^{-1}

The peaks at 466 cm^{-1}, 798 cm^{-1} and 545 cm^{-1} indicated the presence of C-C bond. The peak at 1064 cm^{-1} confirmed the presence of an aromatic group. The presence of uronic acids confers a negative charge and acidic property to the polymer (Jain *et al.*, 2005). Thus, the FTIR spectrum reveals that the EPS is complex acidic polysaccharide.

Chapter IV

Environmental and soil factors influencing the *R. solanacearum* infection

4.1. Introduction

R. solanacearum causing BW is responsible for severe losses to many important crops (mostly solanaceous crops) in tropical and subtropical regions (Hayward, 1991). *R. solanacearum* is classified into four phylotypes, six biovars, five races and many strains (Xue *et al.*, 2011). However, little is known about its distribution and persistence in natural reservoirs (Cook and Sequeira, 1994). Population dynamics of *R. solanacearum* is strongly influenced by various environmental factors as it can multiply nutrient deficient conditions and able to survive in natural environment (Van Elsas *et al.*, 2005; Coutinho, 2005) soil moisture, temperature, pH, salt content and native soil microbiota (Hayward, 1991).

Infection of bacterium primarily initiated in the roots via contaminated soil, irrigation water, or working tools (Lopez and Biosca, 2005; Alvarez *et al.*, 2010). *R. solanacearum* can be disseminated through soil and water, where it can survive for a long time (Lopez and Biosca, 2005; Alvarez *et al.*, 2008; Alvarez *et al.*, 2010) without host plant in soil. It proliferates in the rhizosphere of suitable host plants. Once it reaches inside the host, it colonizes in the xylem portion of plant system and collapses the plant causing wilting (Vasse *et al.*, 1995; Alvarez *et al.*, 2010) and resulting in outbreaks of the disease. Champoiseau *et al.* (2009) reported that the pathogen survival is greatest in wet and well-drained soils, whereas, Buddenhagen and Kelman (1964) demonstrated that its survival is

affected adversely by soil desiccation and by flooding. Abdullah *et al.* (1983) concluded that high soil moisture accumulated as a result of high water table or heavy rainfall usually favours *R. solanacearum* development of bacterial wilt. Temperature is the most important factor responsible for the pathogen survival, increase in ambient temperature (30 to 35˚C) increases the incidence of BW on hosts such as tomato, for many but not all strains of the pathogen (Hayward, 1991).Van Elsas *et al.* (2001) reported that the survival of *R. solanacearum* was strongly dependent on temperature irrespective of inoculum density and physiological state, with maximum survival occurring at 12˚C, 20˚C and 28˚C.

It has been reported that *R. solanacearum* can survive up to 2 years in bare soil under temperate conditions (Shamsuddin *et al.*, 1979). Recently, influence of extreme temperatures and native microbiota on the survival of a *R. solanacearum* biovar 2 in Spain rivers has been studied (Caruso *et al.*, 2005; Àlvarez *et al.*, 2007). Furthermore, Van Overbeek *et al.* (2004) reported that low temperature induced viable-but-nonculturable state of *R. solanacearum* had a role to play in bacterial virulence. Van Elsas *et al.* (2001) reported that survival of *R. solanacearum* biovar 2 strain 1609 was affected when it was incubated in water containing 100 mM NaCl, 10 mM $CaCl_2$ and 3.3 mM $MgSO_4$. This also indicates that *R. solanacearum* cells are stressed by exposure to low water activity. *R. solanacearum* can survive for long periods of time in a nutrient-depleted environment (Grey and Steck, 2001). Nutrient concentration in soil was found affecting bacterial growth and disease development in plant (Lucas, 1975; Kang *et al.*, 2004).

In this study the experiments were designed to set up pilot studies to determine the effect

of pH, NaCl, moisture, temperature on the survival and propagation of the *R. solanacearum* strain, Rs-08-17 Race 1 Phylotype I biovar 3. It is utmost important to understand the ecology of this pathogen which would be helpful in designing low-cost soil management strategies for the control of BW.

4.2. Materials and methods

Bacterial isolate used in this study: *R. solanacearum* Rs-08-17 Race 1 Phylotype I biovar 3.

Soil used for *in vivo* experiments: Nursery soil from glasshouse, ICAR, Research Complex for Goa, Goa.

Media used: CPG medium (composition mentioned in Chapter II) amended with TZC used for *in vitro* assays; SMSA medium (Engelbrecht, 1994; Appendix A) used for all the *in vivo* experiments, King's B medium (composition mentioned in Chapter II) used for enumerating the total bacterial count.

4.2.1. Determination of antibiotic resistant profile of *R. solanacearum* Rs-08-17 isolate

Since soil contains numerous bacteria and other organisms, antibiotic resistant profile of *R. solanacearum* Rs-08-17 isolate was determined to study its population in the inoculated soil. 100µL of *R. solanacearum* Rs-08-17 culture (8 log CFU mL^{-1}) was plated on CPG medium amended with antibiotics. Fourteen antibiotics viz. Amikacin, Ampicillin, Amoxycillin, Nalidixic acid, Kanamycin, Erythromycin, Carbenicillin, Cephatoxime, Tetracycline, Streptomycin, Chloramphenicol, Gentamycin, Penicillin and Bacitracin at working concentrations of 50 µg mL^{-1} and 100 µg mL^{-1} were used. The inoculated plates were incubated at 28±2°C and the growth of the pathogen was

monitored up to 96 h of inoculation.

4.2.2. Soil sample analysis

Nursery soil obtained from Plant pathology Glasshouse, ICAR, Research Complex for Goa, Goa was used for studying its physical and the chemical characteristics. Soil sample were sieved through 2 mm and the unsieved portion was transferred back for pounding again. Soil pH, EC, soil texture, nitrogen, phosphorus and potassium contents was determined using the standard protocols.

4.2.3. Effect of NaCl on the survival of Rs-08-17

4.2.3.1. *In vitro* study

To study the effect of NaCl on growth of *R. solanacearum Rs*-08-17 *in vitro*, TZC agar was amended with different concentrations of NaCl (0.02, 0.04, 0.06, 0.08, 0.1, 0.2 M) after autoclaving. CPG medium (no NaCl) served as a control treatment. Rs-08-17 population was adjusted to 8 log CFU mL^{-1} and 100µL of the culture suspension was spread plated on TZC agar plates and incubated at 28±2°C for 48 h. Number of colonies was recorded at the end of incubation period. The experiment was carried out with two replications.

Effect of NaCl on growth of Rs-08-17 was also evaluated in CPG broth. 5 mL of CPG broth was amended with different concentrations of NaCl (as mentioned above) in 50 mL test tubes. 100µL of *R. solanacearum* (8 log CFU mL^{-1}) was inoculated in to each tube and incubated at room temperature for 60 h on a rotary shaker maintained at 150 rpm. 0.1 mL of the culture sample was taken at every 12 h of time-interval (12, 24, 36, 48 and 60 h) to determine the number of *R. solanacearum* colonies. The experiment was carried out in three replications.

4.2.3.2. Multiplication of Rs-08-17 *in vivo*

To further determine the influence of NaCl on the pathogen population under natural environments bacterial survival was assessed at varying concentrations of NaCl (as mentioned above) in soil. 1 M NaCl (Appendix B.2) stock solution was gradually added to 100 g of sterile soil in aqueous solution to reach the desired NaCl levels. The bacterial culture was suspended in 1X PBS and the bacterial density was adjusted to 6 log CFU mL^{-1}. After 1 h, 10 mL of Rs-08-17 suspension was added and the soil was further incubated at 28°C for 28 days; moisture content of 15-25% was maintained throughout the experiment. Soil sample was taken from the treatments after 7, 14, 21 and 28 days of Rs-08-17 inoculation. The soil samples were thoroughly mixed and analyzed for pathogen population by plating the dilutions on SMSA medium. Experiment was carried out in two replications per treatment.

A similar experiment was conducted to study the effect of NaCl on the pathogen survival in the presence of host (eggplant). The experimental set up and conditions were maintained same as mentioned above. The soil was loosened before the addition of NaCl solution. 25 day old eggplant seedlings were transferred in the pots containing 2 kg of soil. A total of 5 eggplants per pot were maintained in two replications. Population of Rs-08-17 in rhizosphere region was assessed after 7, 14, 21 and 28 days of Rs-08-17 inoculation as described above and the incidence of bacterial wilt was recorded. Two replications per treatment were maintained in the experiment.

4.2.4. Effect of temperature on the survival of Rs-08-17

4.2.4.1. *In vitro* study

To measure the effect of temperature on the growth of *R. solanacearum Rs*-08-17 *in*

vitro, 12 h old Rs-08-17culture (100 µL) with 6 log CFU mL^{-1} population was spread plated on TZC medium incubated at 45°C, 42°C, 37°C, 30°C and 20°C in a temperature controlled chamber for 48 h. Number of colonies was recorded at the end of 24 and 48 h incubation period. The experiment was carried out in two replications per treatment.

Effect of temperature on the growth of Rs-08-17 was also evaluated in CPG broth. Five mL of CPG broth was inoculated with 100µL of *R. solanacearum* (6 log CFU mL^{-1}) in each tube and incubated at the desired temperature up to 48 h on a rotary shaker maintained at 150 rpm. Number of colonies was recorded after 24 and 48 h by plating 100 µL of culture broth. The experiment was carried out in two replications per treatment.

4.2.4.2. Multiplication of Rs-08-17 *in vivo*

To further determine the influence of temperature on the pathogen population under natural environments bacterial survival was assessed at different temperatures (as mentioned above) in soil. The bacterial culture was suspended in 1X PBS and the bacterial cell density was adjusted to 8 log CFU mL^{-1}. 200g of sterile soil per pot was inoculated with 12.5 mL of Rs-08-17 inoculum and moisture content was maintained in the range of 15-25% with regular watering. Soil sample was taken from the treatments after 12, 24, 36 and 48 hours of Rs-08-17 inoculation and analyzed for pathogen population by plating the dilutions on SMSA. Three replications per treatment were maintained in the experiment.

A similar experiment was conducted to study the effect of temperature on the pathogen in the presence of host (eggplant). The experimental set up was similar to the above mentioned one. 25 day old eggplant seedlings were transferred in the pots containing 2 kg

of soil, to which 12.5 mL of pathogen suspension was added. A total of 5 eggplants per pot were maintained in two replications. All the pots were placed in temperature controlled chamber. Total bacterial and population of Rs-08-17 in rhizosphere region was assessed after 7, 14, 21 and 28 days as described above. Incidence of bacterial wilt was also recorded. Two replications were maintained in the experiment.

4.2.5. Effect of pH on the survival of Rs-08-17

4.2.5.1. *In vitro* study

To measure the effect of pH on the growth of *R. solanacearum Rs*-08-17*in vitro*, CPG agar medium was adjusted to different pH values (5.0, 6.0, 7.0, 8.0, 9.0) with 1N HCl (for acidic pH) and 1N NaOH (for alkaline pH) after autoclaving. Rs-08-17 population was adjusted to 8 log CFU mL^{-1} and 100 µL of the culture suspension was spread plated on CPG agar (with varying pH) plates and incubated at 28±2°C for 48 h. Number of colonies was recorded at the end of incubation period. The experiment was carried out in two replications.

Effect of pH on growth of Rs-08-17 was also evaluated in CPG broth. 5 mL of CPG broth was adjusted with different pH values (as mentioned above) in 50 mL test tubes. 100 µL of *R. solanacearum* (8 log CFU mL^{-1}) was inoculated in to each tube and incubated at room temperature for 60 h on a rotary shaker maintained at 150 rpm. 0.1 mL of the culture sample was taken at every 12 h of time-interval (12, 24, 36, 48 and 60 h) to determine the number of *R. solanacearum* colonies. The experiment was carried out in three replications.

4.2.5.2. Multiplication of Rs-08-17 *in vivo*

Nursery soil pH was adjusted to values (pH 5.0, 6.0, 7.0, 8.0). Ammonium sulfate was used to lower the soil pH whereas; Calcium carbonate was used to make the soil alkaline. Four kilograms of soil per pot was inoculated with 250 mL of Rs-08-17 inoculum and moisture content was maintained in the range of 15-25% with regular watering. The bacterial culture was suspended in 1X PBS and the bacterial density was adjusted to 6 log CFU mL^{-1}. Rs-08-17 and total bacterial population in soil were analyzed after 7, 14, 21, and 28 days of inoculation at two depths (0-10 cm and 10-20 cm from the soil surface). Soil sample was taken from the treatments and spread plated on SMSA (for Rs-08-17 population) and King's B medium (for total bacterial count). The number of colonies was recorded and three replications per treatment were maintained in the experiment.

A similar experiment was conducted to study the effect of pH on the pathogen in the presence of host (eggplant). The experimental set up was similar to the above mentioned one. 25 day old eggplant seedlings were transferred in the pots containing 2 kg of soil. A total of 5 eggplants per pot were maintained in three replications. Total bacterial and pathogen population of Rs-08-17 in rhizosphere region was assessed after 7, 14, 21 and 28 days of Rs-08-17 inoculation as described above and the incidence of BW was recorded. Three replications per treatment were maintained in the experiment.

4.2.6. Effect of moisture on the survival of Rs-08-17 *in vivo*

To investigate soil moisture effect on the pathogen survival, four soil moisture level ranges (0-25%, 26-50%, 51-75%, 76-100%) moisture holding capacity (MHC) were used. 4 kg of soil per pot with varying moisture content was inoculated with 250 mL of Rs-08-17 inoculum adjusted to a population of 6 log CFU mL^{-1}. The moisture content

was maintained according to treatment levels by adding required quantity of water regularly. The loss of water through drainage was also avoided by closing the drain holes in the experimental pots. A total of 5 eggplants per pot were maintained in three replications. Before sampling, the uppermost layer of soil was excluded. Sampling was done at two depths in the soil (0-10 cm and 10-20 cm from the soil surface) using a soil auger after 7, 14, 21, and 28 days of inoculation. Soil sample was taken from the treatments and spread plated on SMSA (for Rs-08-17 population) and King's B medium (for total bacterial count). The number of colonies was recorded and three replications per treatment were maintained in the experiment.

4.2.7. Statistical analysis

Data were analyzed in Statistical Analysis Software version 8.1 (Version 8.1, SAS Institute Inc., Cary). Data were analyzed by ANOVA using the GLM procedure and the coefficient of determination (R^2) was obtained. Specific comparisons of least-square means were evaluated for significance using Tukey's HSD adjusted P-values. The number of CFU per gram of dry soil and CFU per milliliter was log transformed (log10 [x + 1]) for statistical analysis (Steel and Torrie, 1960). For non-detectable *R. solanacearum* populations transformed values of zero were used for calculation of the treatment means. Analysis of variance (ANOVA) was carried out for each sampling date and the differences in pathogen population was analyzed for various factors (NaCl, temperature, pH, MHC) (Swallow, 1984).

4.3. Results and Discussion

4.3.1. Determination of antibiotic resistant profile of *R. solanacearum* Rs-08-17 isolate

Rs-08-17 isolate was found to be resistant to 3 antibiotics viz. Chloramphenicol, Penicillin and Bacitracin at 50 µg μL^{-1} (Table 4.1).

Table 4.1 Antibiotic sensitivity profile for *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate

No.	Antibiotic	50 µg/µl	100 µg/µl
1	Amikacin	-	-
2	Ampicillin	-	-
3	Amoxycillin	-	-
4	Nalidixic acid	-	-
5	Kanamycin	-	-
6	Erythromycin	-	-
7	Carbenicillin	-	-
8	Cephatoxime	-	-
9	Tetracycline	-	-
10	Streptomycin	-	-
11	Chloramphenicol	+++	++
12	Gentamycin	-	-
13	Penicillin	+++	+
14	Bacitracin	++	++

-, No growth; +++, highest growth; ++, moderate growth; +, mild growth

4.3.2. Soil analysis

Glasshouse nursery soil was used for all the greenhouse studies. BW has been reported earlier in this soil and therefore, its physical and chemical characteristics were recorded as given in Table 4.2. Soil characteristics and its nutrient concentration are known to be involved in disease development or suppression (Lucas, 1975) and were therefore recorded. Organic carbon was estimated as 1.93% in the nursery soil. Nitrogen content in the soil was 173 kg ha^{-1}. Phosphate content was estimated to about 37.6 kg ha^{-1} in the nursery soil.

Table 4.2 Major properties of nursery soil used in the study

Soil properties	Nursery soil
Soil texture	Sandy
pH	6.86
EC (µs)	0.054
[a]organic carbon (%)	1.93
[b]Potassium (kg ha^{-1})	642
[c]Phosphate (kg ha^{-1})	37.64
[d]Nitrogen (kg ha^{-1})	173.6

EC Electrical conductivity
[a]Using Wet oxidation method
[b]Using ammonium acetate method and read by flame photometer
[c]Using Olsen's Method
[d]Using alkaline potassium permanganate method

It has been reported in the past that BW diseases were suppressed by presence of certain organic contents in some soils (Gorissen *et al.*, 2004; Islam and Toyota, 2004; Satoh and Toyota, 2004). There are reports stating the highest *R. solanacearum* survival in soil with organic matter content of 2.0-2.5% (Van Elsas *et al.*, 2000). Michel and Mew (1998) has reported earlier that presence of high nitrogen content reduced BW in sandy soils. This was also supported by Lucas (1975) who reported that high concentration of nitrate depresses the growth of *R. solanacearum* and wilt development in tobacco. Recently, Lemaga *et al.* (2005) proved that 100 kg ha^{-1} has significantly decreased BW incidence on potato. However, Messiha *et al.* (2006) has reported a significant positive correlation between nitrogen content, disease severity and wilt incidence. However, increased phosphate content of soil was found to negatively correlate to the disease incidence (Kang *et al.*, 2004).

4.3.3. Effect of NaCl on the survival Rs-08-17

4.3.3.1. *In vitro* study

When the *R. solanacearum* population was assessed at varying NaCl concentrations on TZC agar it was observed that the population was 9 log CFU mL^{-1} for all the treatments except at 0.1 M NaCl where it decreased significantly to 8 log CFU mL^{-1} (Fig. 4.1). *R. solanacearum* failed to survive at 0.2 M NaCl concentration. NaCl concentration also had an effect on the phenotypic characteristics of the *R. solanacearum*. With increase in NaCl concentration, the size of the colony and EPS production decreased.

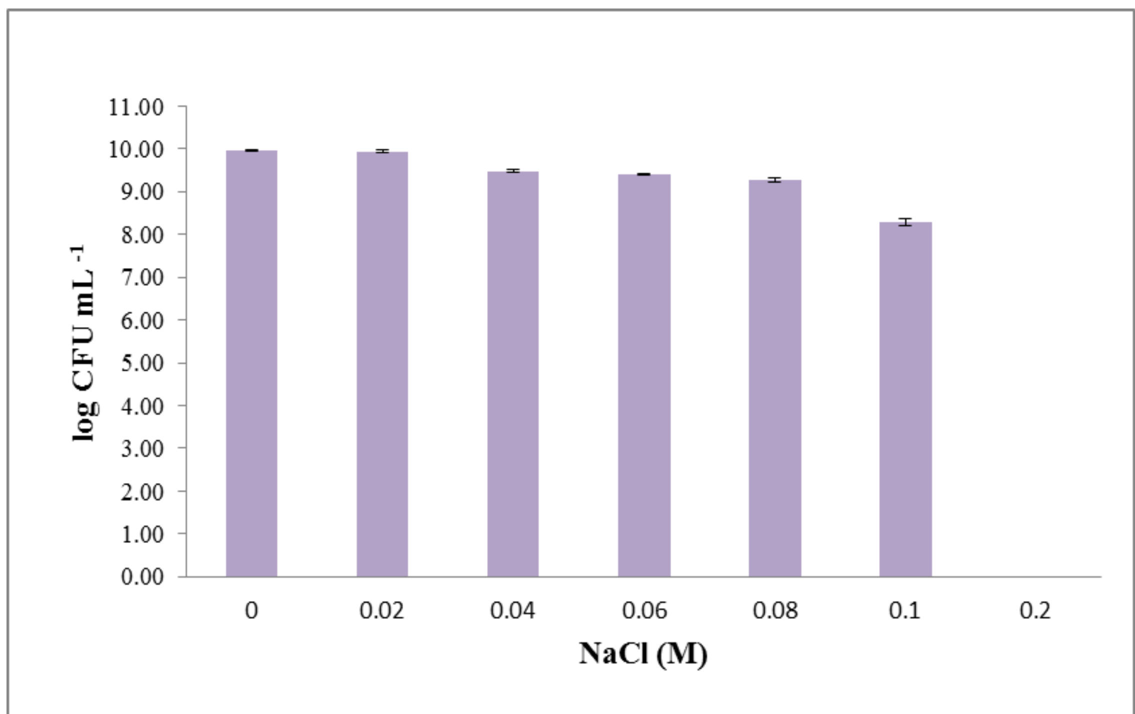


Fig. 4.1 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate on TZC medium amended with different NaCl concentrations. Initial pathogen population: 8 log CFU mL^{-1}; Time of incubation: 0-48 h. Data shown reflects the average of two experiments, each containing two plate counts. Bars indicate standard error of the mean.

In CPG broth, Rs-08-17 population was higher in the control medium (without NaCl). A drastic decline in the *R. solanacearum* population was observed from 0.02 M to 0.2 M NaCl concentrations with 2 log CFU mL^{-1} difference within 12 h. However, a slight

increase in the *R. solanacearum* growth was recorded at 0.02 M and up to 0.06 M NaCl treatment after 48 h. Rs-08-17 population remained almost constant at 0.08 M NaCl throughout the period where as in 0.1 M and 0.2 M NaCl treatments, the population remained constant up to 48 h and gradually decreased there after (Fig. 4.2).

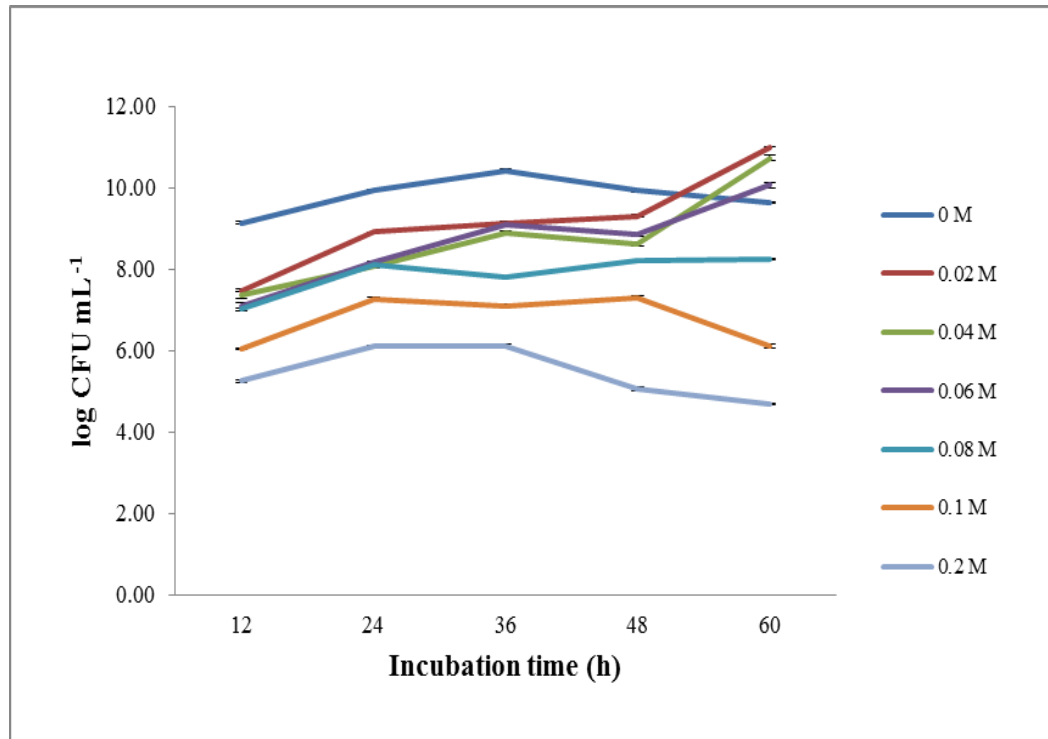


Fig. 4.2 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate in CPG broth amended with different NaCl concentrations over a 60 h period. Initial pathogen population: 8 log CFU mL^{-1}; Time of incubation: 0-60 h; Each line represents the mean value of three replicates, each containing two plate counts. Bars indicate standard error of the mean

4.3.3.2. Multiplication of Rs-08-17 *in vivo*

In soil, the *R. solanacearum* population gradually decreased with the increase in incubation period. The *R. solanacearum* population decreased from 4 log CFU g^{-1} to 1 log CFU g^{-1} in case of control, 0.02 M and 0.04 M NaCl treatments within 28 days of inoculation. After every 7 days' time period there was a decrease of 1 log CFU g^{-1} population in all the treatments. Interestingly, it was noted that Rs-08-17 failed to survive

in treatments beyond 0.04 M NaCl and above (Table 4.3; Fig. 4.3) in between 21 to 28 days post inoculation.

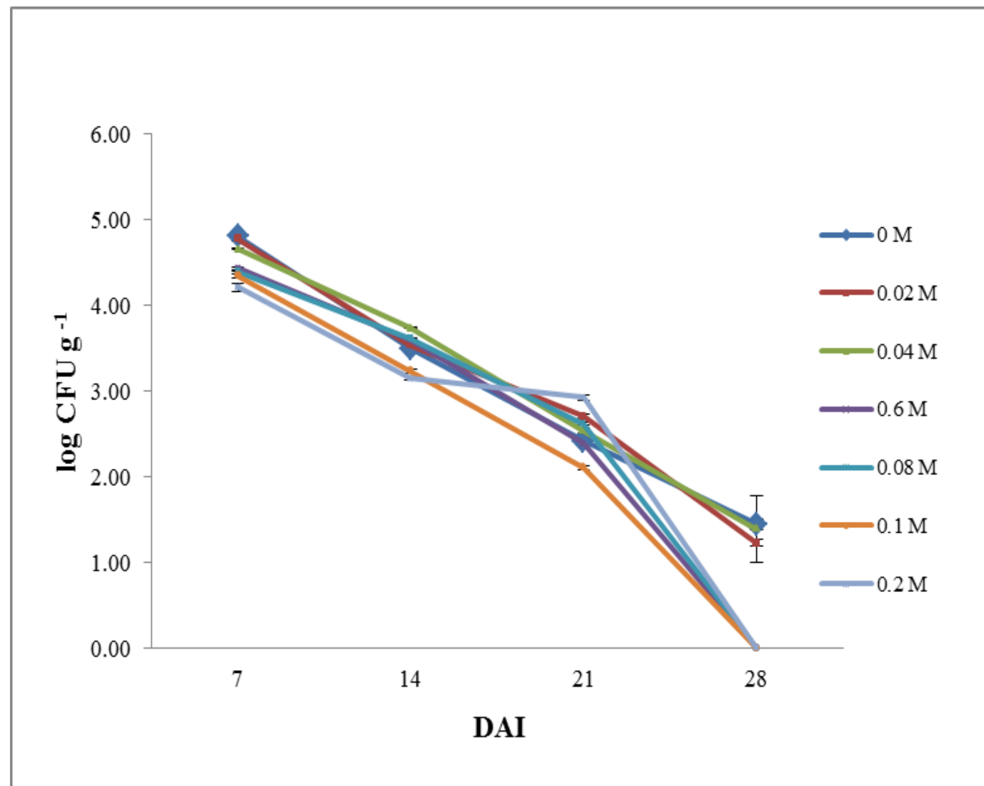


Fig. 4.3 Population of *R. solanacearum* Race 1 biovar 3 isolate Rs-08-17 at different NaCl treatments in soil. Initial pathogen population: 6 log CFU mL^{-1}; Time of incubation: 0-28 days. Viable cells were enumerated by dilution plating on SMSA agar. This experiment was repeated two times; Points represent means of all subsamples; bars indicate standard error.

It was observed that the *R. solanacearum* successfully multiplied up to 0.04 M NaCl concentration within 7 days of inoculation and increased by 1 log CFU g^{-1} in the rhizosphere region of eggplant. However, at 0.1 M and 0.2 M NaCl treatments the *R. solanacearum* population decreased from 6 log CFU g^{-1} to 4 log CFU g^{-1} (Table 4.3; Fig. 4.4). *R. solanacearum* population declined in the rhizosphere after 14 days of inoculation in case of 0.06 M to 0.2 M NaCl treatments and also after 21 days in case of control, 0.02 M and 0.04 M NaCl treatments. Overall, the *R. solanacearum* population in the rhizosphere was maintained approxiamately at 6-7 log CFU g^{-1} in case of lower NaCl

treatments (i.e. up to 0.04 M), whereas, it reduced drastically to < 2 log CFU g^{-1} in higher NaCl treatments. Initially 40% of wilt was recorded in all the treatments after 7 days of inoculation period. Highest number of plants (60%) wilted in the control and 0.08 M NaCl treatment than in any other treatments after 14 days of *R. solanacearum* inoculation (Table 4.3).

Table 4.3 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at varying NaCl treatments in soil and in the rhizosphere region of host

NaCl (M)	**Soil* (log CFU g^{-1})**				**Rhizosphere region* (log CFU g^{-1})**				**Wilt Incidence (%)**			
	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI
0	4.80±0.004	3.49±0.022	2.41±0.041	1.44±0.062	7.71±0.025	7.70±0.077	7.56±0.006	6.63±0.005	50	60	70	70
0.02	4.77±0.022	3.54±0.022	2.70±0.022	1.22±0.044	7.73±0.011	7.51±0.020	7.65±0.019	6.13±0.054	40	40	60	60
0.04	4.65±0.010	3.73±0.006	2.53±0.032	1.38±0.389	7.66±0.017	7.72±0.008	7.52±0.026	6.17±0.058	40	40	50	60
0.6	4.43±0.018	3.59±0.006	2.38±0.027	0.0	6.40±0.037	6.61±0.058	4.42±0.008	4.47±0.022	40	40	40	40
0.08	4.39±0.000	3.61±0.006	2.61±0.016	0.0	6.44±0.015	6.62±0.016	3.64±0.019	2.47±0.031	50	60	60	60
0.1	4.34±0.023	3.23±0.022	2.10±0.026	0.0	4.39±0.045	4.23±0.020	2.24±0.020	1.64±0.020	40	40	40	40
0.2	4.21±0.051	3.14±0.017	2.92±0.031	0.0	4.26±0.033	4.18±0.040	1.90±0.030	0.75±0.151	40	40	40	40

Initial Inoculum: 6 log CFU mL^{-1}; Incubation period: 7, 14, 21 and 28 days; NaCl concentrations in Molar (0.02, 0.04, 0.06, 0.08, 0.1 and 0.2 M); DAI- Days after inoculation

The data are presented as the means of two replicates each containing three subsamples; ± Standard error

Culturable cells were enumerated at regular intervals by dilution plating on SMSA medium

* Pathogen population in soil and rhizosphere region was statistically significant at different NaCl treatments and incubation time ($P < 0.0001$); $R^2 = 0.9$

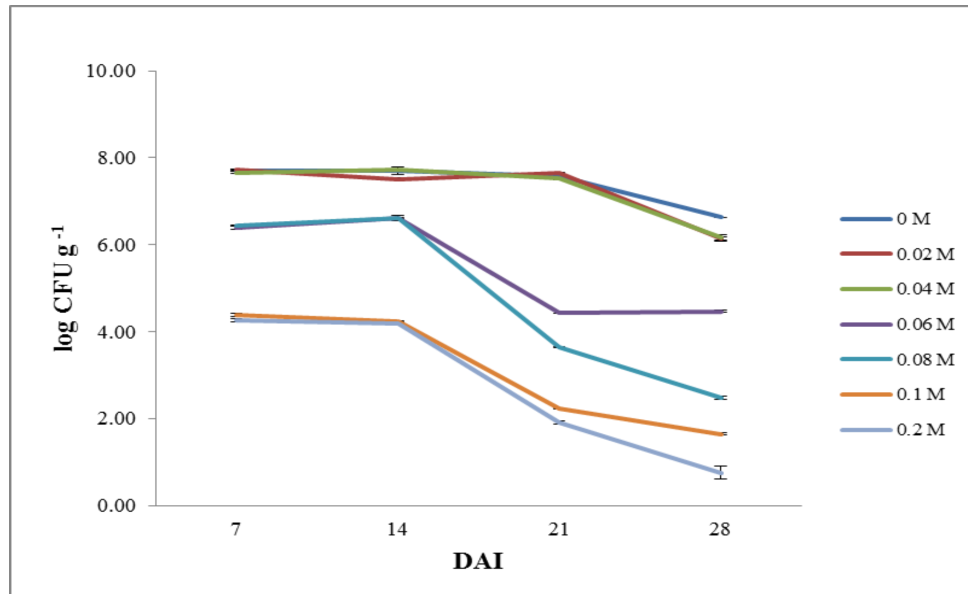


Fig. 4.4 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at different NaCl treatments in rhizosphere region of the host. Initial pathogen population: 6 log CFU mL^{-1}; Experiment was monitored up to 28 days of time period. Viable cells were enumerated by dilution plating on SMSA agar. Each experiment contained two replicates, each containing three subsamples. Points represent means of all subsamples; bars indicate standard error.

There was no correlation between the wilt incidence and the NaCl treatments. The statistical data analysis proved that the change in *R. solanacearum* population was significant when NaCl treatments and incubation time varied.

Salts are known for its importance in the sustenance of all microorganisms and are essential in their survival. A previous finding showed that levels of sea water salts realistic for drainage water in coastal areas were detrimental to *R. solanacearum* survival (Van Elsas *et al.*, 2001). This indicates a severe detrimental effect of the salt concentration on the *R. solanacearum* during its active propagation. The results of this study are in conformity with a previous report on *R. solanacearum* biovar 2 strain 1609 survival was upon incubation in water containing 100 mM NaCl (Van Elsas *et al.*, 2001). There are reports that levels of seawater salts similar to those of water in coastal areas inhibited the surviving of the bacterium due to osmotic tensions (Van Elsas *et al.*, 2001;

2005). In soil experiments, *R. solanacearum* population was enumerated on SMSA medium than in that on TZC for selectivity of the *R. solanacearum* from the soil. The medium was used by several authors for studying the ecology of plant *R. solanacearum* (Granada and Sequeira, 1983; Elphinstone *et al.*, 1997; Ito *et al.*, 1998).

4.3.4. Effect of temperature on the survival of Rs-08-17 *in vitro* and *invivo*

4.3.4.1. *In vitro* study

In this study, *R. solanacearum* multiplied at 30˚C and 37 ˚C only. However, the *R. solanacearum* population started reducing at 20 °C from 6 log CFU mL^{-1} to 3.5 log CFU mL^{-1} after 24 h of incubation. Rs-08-17 did not survive at temperature above 37˚C (Fig. 4.5).

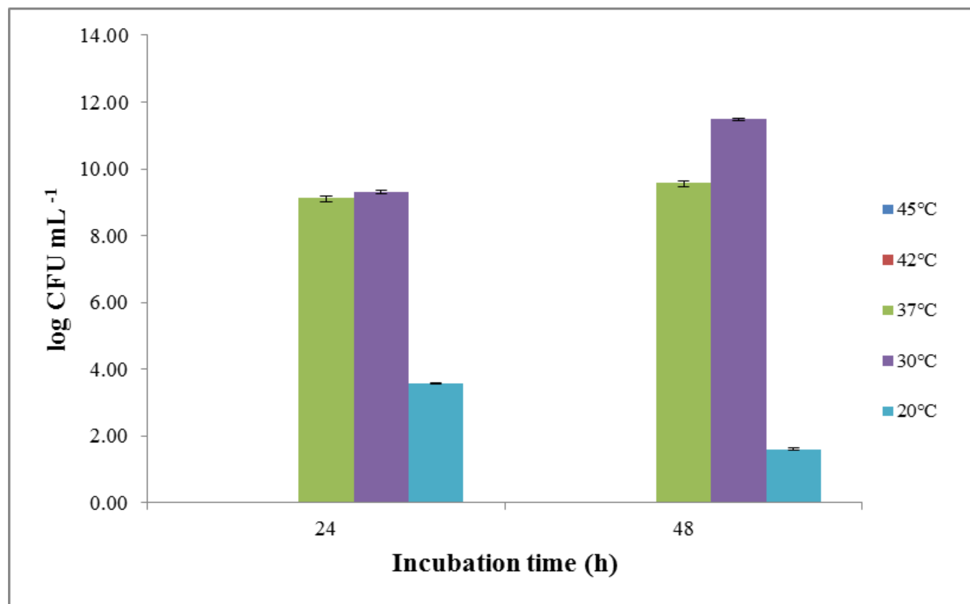


Fig. 4.5 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at different temperatures on TZC medium. Initial pathogen population: 6 log CFU mL^{-1}; Time of incubation: 0-48 h. Data shown reflects the average of two experiments, each containing two plate counts. Bars indicate standard error of the mean

In CPG broth, Rs-8-17 did not survive at 42˚C and 45˚C. Optimum growth was observed at 30˚C compared to other temperatures. Highest population was recorded after 36 h of

incubation at 30 ˚C. Rs-08-17 growth started decreasing at 20 ˚C which was completely inhibited within 60 h of incubation (Fig. 4.6).

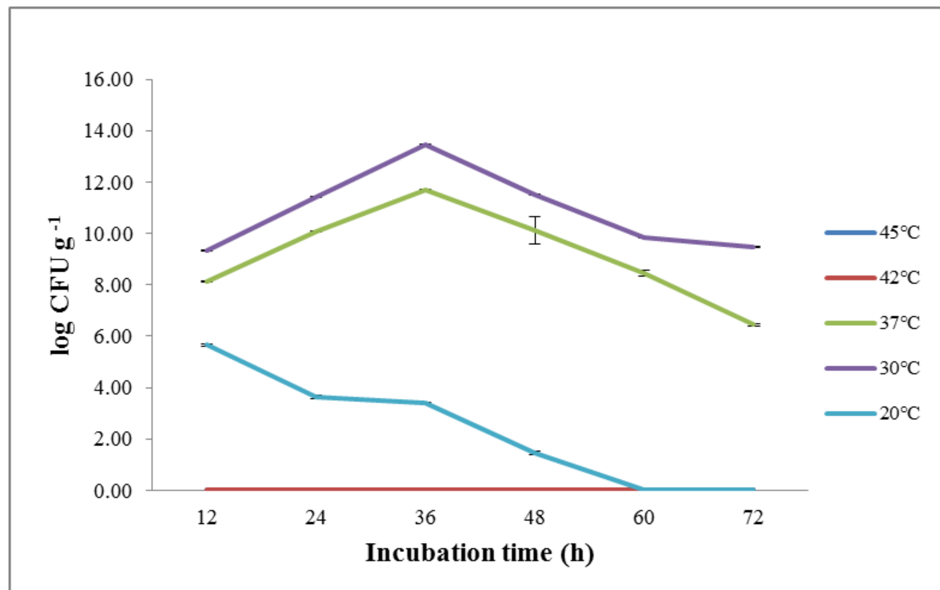


Fig. 4.6 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate in CPG broth over a 72 h period at different temperatures. Initial pathogen population: 6 log CFU mL^{-1}. Each line represents the mean value of two replicates, each containing two plate counts. Bars indicate standard error of the mean

4.3.4.2. Multiplication of Rs-08-17 *in vivo*

The total *R. solanacearum* population inoculated in the soil was 8 log CFU g^{-1}. Reduction in the *R. solanacearum* population was observed in the range 8 log CFU g^{-1} to 4 log CFU g^{-1}at 42°C and 45°C respectively, within 12 h of incubation (Fig. 4.7). *R. solanacearum* survival was significantly reduced from 8 log CFU g^{-1} to 1 log CFU g^{-1} in the samples at 42°C and 45°C from 0-36 h, respectively (Table 4.4). At 20°C, Rs-08-17 population decreased from 8 log CFU g^{-1} to 3 log CFU g^{-1} within 48 h of incubation period.

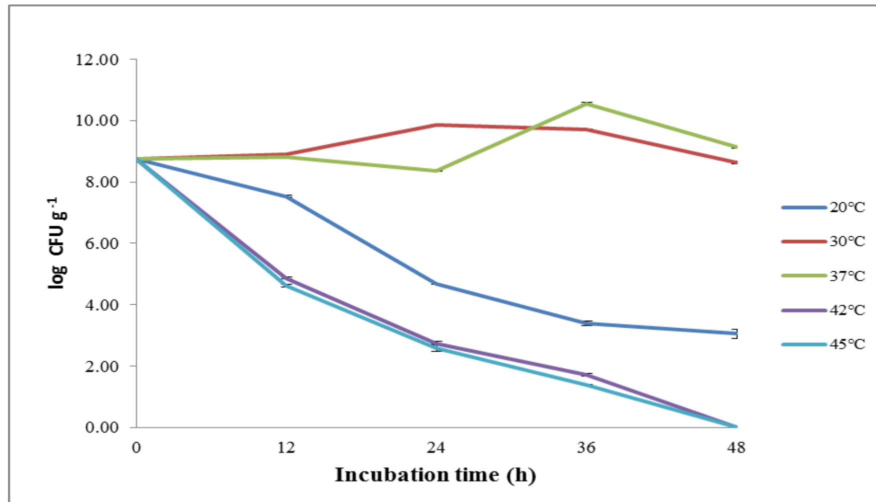


Fig. 4.7 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at different temperatures in soil. Initial pathogen population: 8 log CFU mL^{-1}; Time of incubation: 0-48 h. Colonies was enumerated at regular intervals by dilution plating on SMSA agar plates. This experiment was repeated three times. Each experiment contained two replicates, each containing three subsamples. Points represent means of all subsamples; bars indicate standard error

It has been reported that Race 1 strains can survive longer in soil, occur in warmer regions (26-36°C) and have a wider host range (Hayward, 1991). French (1994) observed that soil survival of *R. solanacearum* isolate R3bv2A was inhibited at average soil temperatures of 14°C or below in the tropical highlands. The results indicated that Rs-08-17 in wet soil exposed to a constant and continual temperature of 42°C, 45°C and above had lost their viability after 48 h. The *R. solanacearum* multiplied at a faster rate at 30°C and 37 °C throughout the experiment (Fig. 4.7). However, there are reports of *R. solanacearum* survival up to 2 years in bare- or weed-followed soil under temperate conditions (Shamsuddin *et al.*, 1979).

Table 4.4 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at varying temperatures treatments in soil and rhizosphere region of host

Time (h)[a]	**Soil* (log CFU g^{-1})**					Time (DAI)[b]	**Rhizosphere region* (log CFU g^{-1})**					**Wilt Incidence (%)**				
	20 °C	30 °C	37 °C	42 °C	45 °C		20 °C	30 °C	37 °C	42 °C	45 °C	20°C	30°C	37°C	42°C	45°C
0	8.7±0.02	8.7±0.02	8.7±0.02	8.7±0.02	8.7±0.02	0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0					
12	7.5±0.03	8.9±0.01	8.8±0.00	4.8±0.03	4.6±0.04	7	5.5±0.03	7.67±0.00	7.5±0.01	2.1±0.02	2.2±0.09	0	40	40	0	0
24	4.6±0.00	9.8±0.01	8.4±0.02	2.7±0.07	2.6±0.12	14	4.4±0.03	7.63±0.01	7.1±0.02	0.7±0.09	0.92±0.08	0	60	40	0	0
36	3.4±0.09	9.7±0.02	10.5±0.03	1.7±0.04	1.4±0.02	21	4.2±0.06	7.35±0.01	7.0±0.02	0.00	0.00	20	60	40	0	0
48	3.0±0.15	8.6±0.05	9.1±0.04	0.00	0.00	28	2.3±0.03	6.52±0.05	6.6±0.02	0.00	0.00	20	60	40	0	0

Initial Inoculum: 8 log CFU mL^{-1} ; [a]Incubation period: 12, 24, 36 and 48 h; [b]Incubation period: 7, 14, 21 and 28 days; Temperature in °C (20, 30, 37, 42, 45); DAI- Days after inoculation; Culturable cells were enumerated at regular intervals by dilution plating on SMSA medium. The data are presented as the means of two replicates each containing three subsamples; ± Standard error

* Pathogen population in soil and rhizosphere region was statistically significant at different temperature treatments and incubation time ($P < 0.0001$); $R^2 = 0.9$

R. solanacearum population at the rhizosphere region was highest at 30°C followed by 37°C and remained almost constant up to 28 days, whereas, *R. solanacearum* population gradually started decreasing at 20°C (Fig. 4.8).

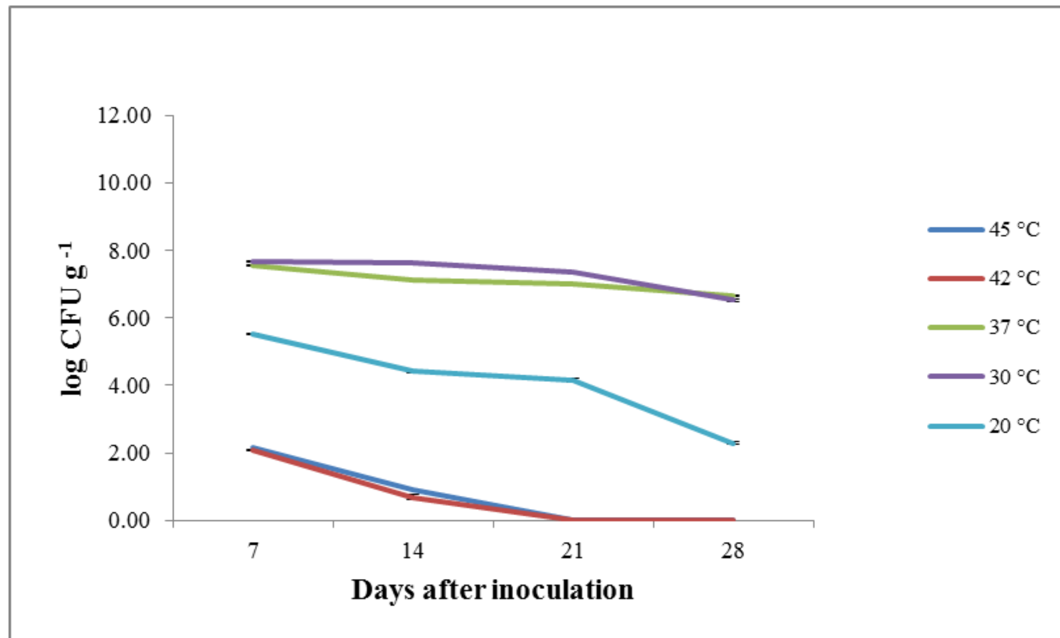


Fig. 4.8 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate at different temperatures in rhizosphere region of the host. Initial pathogen population: 8 log CFU mL^{-1}; Time of incubation: 0-28 days. Colonies were enumerated at regular intervals by dilution plating on SMSA agar plates. This experiment was repeated three times. Each experiment contained two replicates, each containing three subsamples. Points represent means of all subsamples; Bars indicate standard error.

It has been earlier reported that the occurrence of high population densities of *R. solanacearum* could be due to exudation from plant roots (Moffett and Wood, 1984). These findings are in agreement with the results reported by Scherf *et al.* (2010) that the prolonged but constant low-temperature stress appeared to broadly weaken *R. solanacearum* growth. However, contradictory reports were presented by Milling *et al.* (2009) who observed the survival of *R. solanacearum* R3Bvr2 in fallow soils with temperatures as low as 4°C. Earlier, Harris (1976) found that the optimum temperature for R3bv2A was 27°C and the minimum was 12-15°C. In this study, Rs-08-17 did not

survive at 42°C and 45°C even in the rhizosphere region after 7 days of inoculation and completely got suppressed within 21 days post inoculation (Table 4.4; Fig. 4.8).

Wilt incidence was recorded only in 3 treatments viz. 30°C, 37°C and 20°C. Initially 40% of plants were wilted at 30°C and 37°C within 7 days post inoculation of Rs-08-17. This was in agreement to the temperature range which was reported by Zehr (1970). Higher number of plants (60%) wilted at 30°C temperature after 14 days of inoculation, followed by 37°C. At 20°C, only 20% of plants wilted that too after 21 days of inoculation (Table 4.4).

The findings of this study are consistent with the previous reports that mentioned temperature as the most important factor affecting the host *R. solanacearum* interaction as well as their survival in soils (Van Elsas *et al.*, 2000; Milling *et al.*, 2009). In general, increase in ambient temperature to between 30-35°C increases the incidence and rate of onset of BW on hosts in majority of the cases (Hayward, 1991). Graham and Lloyd (1979) reported that survival of *R. solanacearum* (Race 3) was enhanced in deeper soil layers, presumably due to less temperature fluctuation, to a lower degree of predation by protozoa or of competition or antagonism by the indigenous microbiota. Both survival *in vitro* and virulence of *R. solanacearum* are optimal at temperatures between 24 and 35°C (Pradhanang and Elphinstone, 1996; Pradhanang *et al.*, 2000). However, it has been also proved that *R. solanacearum* can grow at temperatures up to 39°C (Hayward, 1976), and higher in temperate aquatic environments (Roszak and Colwell, 1987).

4.3.5. Effect of pH on the survival of Rs-08-17 *in vitro* and *in vivo*

4.3.5.1. *In vitro* study

When the effect of pH on *R. solanacearum* Rs-08-17 survival was studied *in vitro* it was observed that the *R. solanacearum* survived at all the pH values except pH 5.0 on the solid medium (Fig. 4.9). Also it was noted that the size of the colony and eps production had decreased drastically at pH 8.0 and 9.0. Growth of Rs-08-17 was optimum in the control (pH 6.4) as compared to that of pH 7.0.

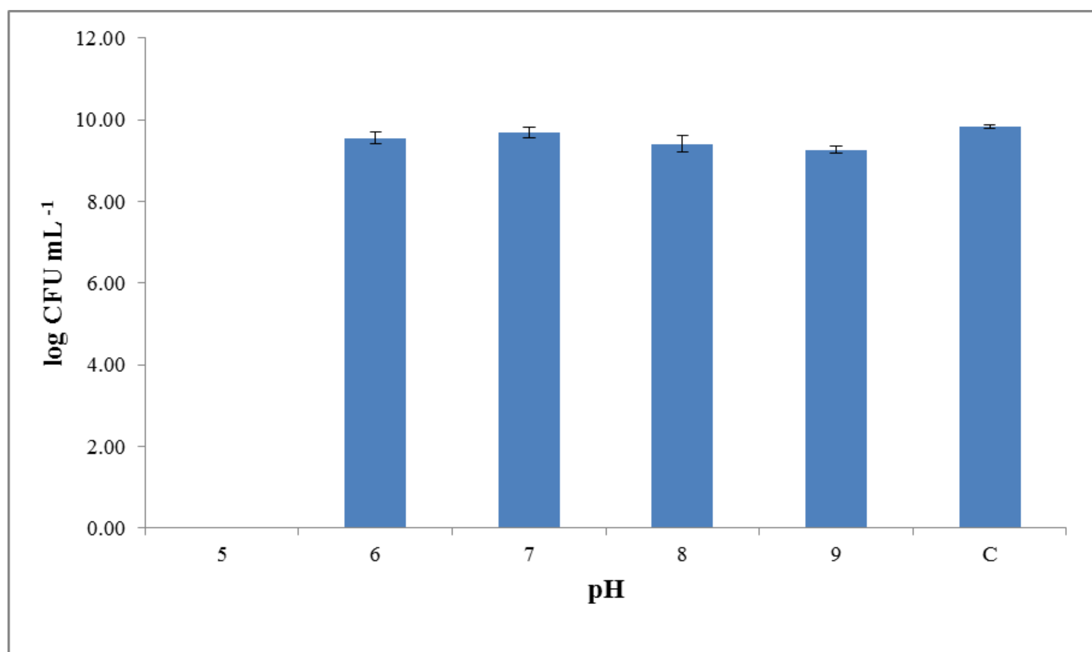


Fig. 4.9 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate on TZC medium adjusted with different pH values. Initial pathogen population: 8 log CFU mL^{-1}; Time of incubation: 24-48 h. Data shown reflects the average of two experiments, each containing two plate counts. Bars indicate standard error of the mean

In CPG broth at various pH values, the bacterium grew rapidly in pH 6.0 and 7.0 followed by pH 8.0 and pH 5.0. Optimum pH for the *R. solanacearum* growth was found to be 6, followed by 7. Maximum growth of Rs-08-17 was recorded after 36 h at all pH values. The *R. solanacearum* population declined drastically between 48 to 60 h of

incubation period. The growth of the *R. solanacearum* at pH 5.0 and pH 9.0 was suppressed and severely reduced (Fig. 4.10).

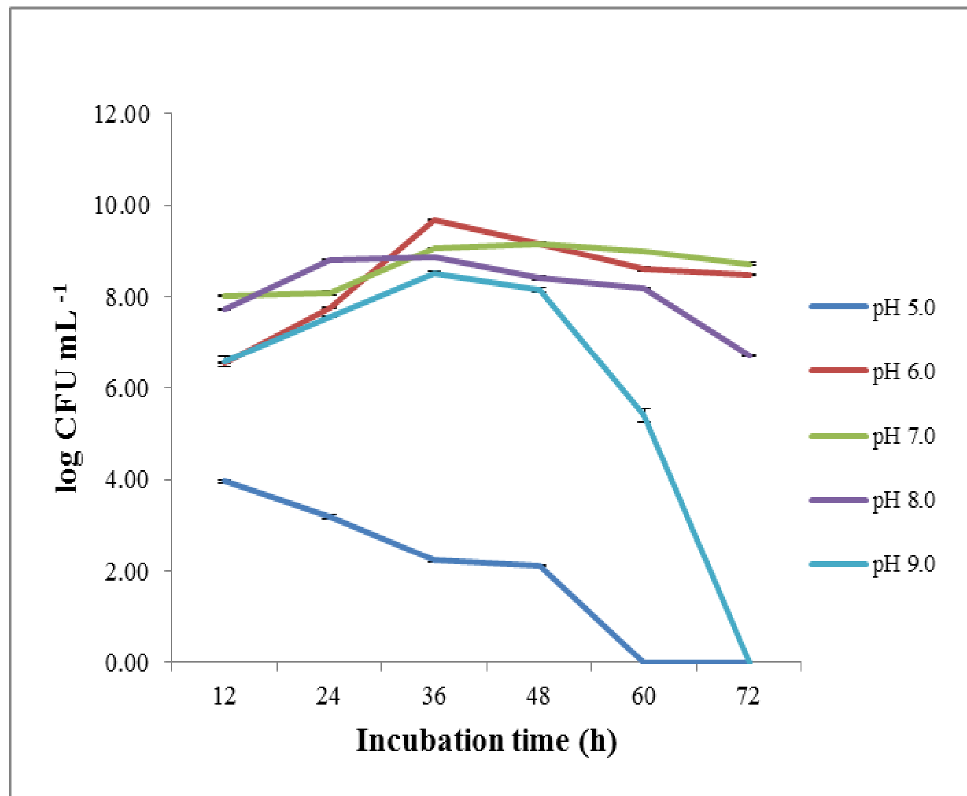


Fig. 4.10 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate in CPG broth adjusted to different pH values over a 72 h period. Initial pathogen population: 8 log CFU mL^{-1}. Each line represents the mean value of three replicates, each containing two plate counts. Bars indicate standard error of the mean

4.3.5.2. Multiplication of Rs-08-17 *in vivo*

There was no significant difference in the population of Rs-08-17 at 0-10 cm and 10-20 cm depth of soil (Fig. 4.11 a, b, d, e). At pH 7.0, the population of *R. solanacearum* was maximum as compared to the other pH treatments. *R. solanacearum* also survived at pH 5.0 and pH 8.0 (Table 4.5).

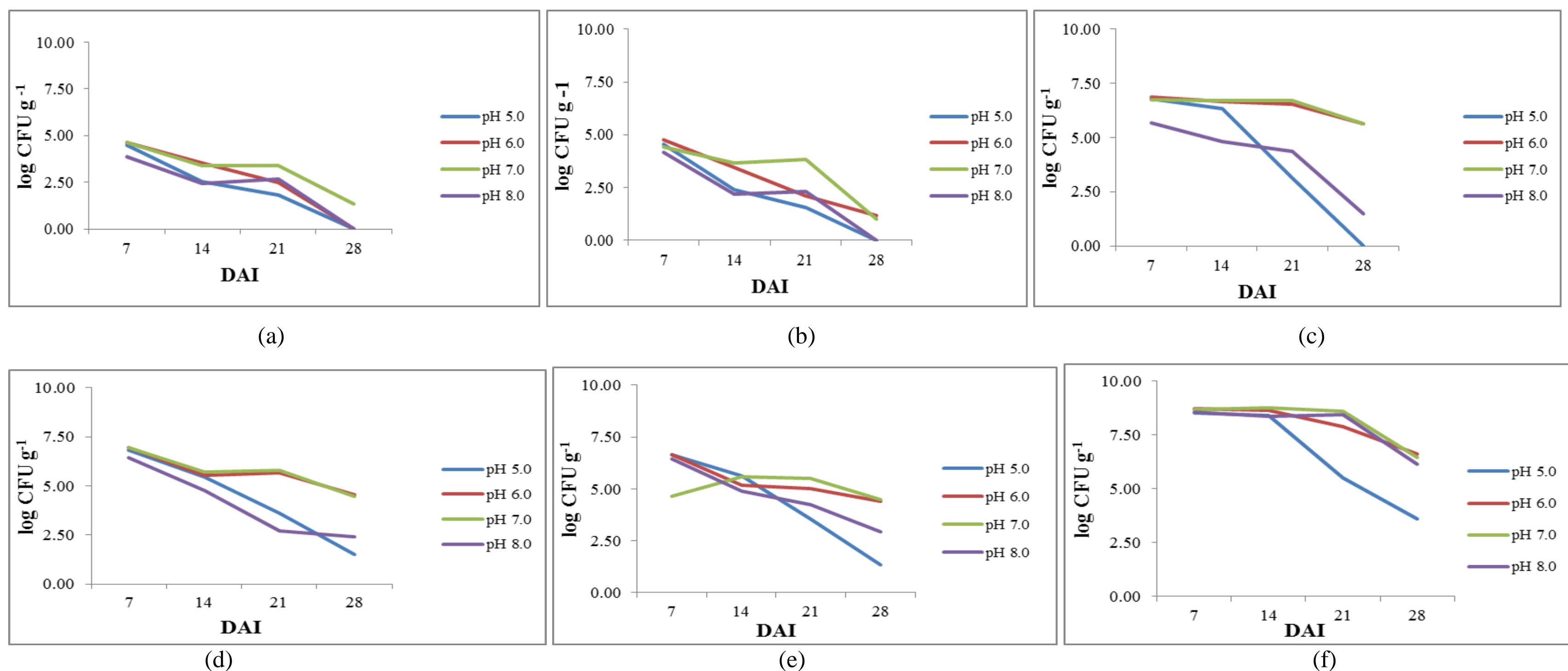


Fig. 4.11 Survival of *R. solanacearum* Race 1 biovar 3 isolate Rs-08-17and soil bacteria under different soil pH treatments. Soil samples removed from (a, d) 0-10 cm depth); (b, e) 10-20 cm depth) and (c, f) rhizosphere region of eggplant. The population of the bacteria was calculated on day 7, day 14, day 21 and day 28 by plating on SMSA (for Rs-08-17 population) and KB medium (for total bacterial population). Data shown reflect the average of three experiments. The mean values of the population were transformed into log CFU g^{-1}

a, b, c- Rs-08-17 Population *in vivo*; d, e, f- Total bacterial population *in vivo*.

There is also strong evidence that soil pH is an important determinant of bacterial diversity and community structure on a global scale (Fierer and Jackson, 2006). This factor has been studied through a combination of physiological and soil microcosm studies. The soil pH was adjusted to pH 5.0 by addition of ammonium sulphate. It has been reported previously by Michel and Mew (1998) that ammonium reduces the growth of *R. solanacearum*, but does not suppress and its effect was found to be pH dependent. The *R. solanacearum* is reported to survive better in deep soil even up to two years (Wenneker *et al*., 1999; Van Elsas *et al*., 2000).

The *R. solanacearum* population was recorded to be highest in the rhizosphere region of the host than in the soil at pH 6.0 and 7.0, *R. solanacearum* survived better at pH 8.0 of soil than pH 5.0 (Fig. 4.11 c). At pH 8.0 the *R. solanacearum* population declined from 4.3 log CFU g^{-1} to 1.4 log CFU g^{-1} in the rhizosphere region within a period of 7 days post. There was a major reduction in the Rs-08-17 population at pH 5.0 from 6 log CFU g^{-1} to 3 log CFU g^{-1} from 14th day to 21st day of inoculation in the rhizosphere region. (Table 4.5). However, there are reports of *R. solanacearum* multiplying in xylem sap of tomato where in the pH is around 5.0 (Wilkinson *et al.*, 1998). Michel and Mew (1998) showed that a high pH may have a deleterious effect on *R. solanacearum* as it restricts the availability of many soil nutrients.

Table 4.5 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate and total soil bacteria at varying pH values in soil and in the rhizosphere region of host

		Sampling site*												Wilt incidence[c] (%)			
		0-10 Cm depth				10-20 Cm depth				Rhizosphere region							
pH*	Source	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI
5.0	Rs[a]	4.48±0.08	2.5±0.08	1.84±0.06	0.00	4.53±0.02	2.4±0.09	1.55±0.09	0.00	6.8±0.03	6.3±0.10	3.14±0.05	0.00	0.0	0.0	0.0	0.0
	TC[b]	6.81±0.05	5.4±0.13	3.61±0.12	1.5±0.06	6.66±0.07	5.6±0.06	3.56±0.07	1.3±0.08	8.5±0.10	8.4±0.16	5.50±0.32	3.61±0.05				
6.0	Rs[a]	4.65±0.04	3.5±0.04	2.50±0.11	0.00	4.74±0.05	3.4±0.04	2.09±0.03	1.2±0.08	6.8±0.08	6.6±0.11	6.52±0.03	5.63±0.03	53.3	66.7	73.3	73.3
	TC[b]	6.93±0.02	5.5±0.06	5.67±0.03	4.5±0.16	6.66±0.08	5.2±0.03	5.03±0.03	4.4±0.11	8.7±0.12	8.6±0.04	7.90±0.01	6.62±0.03				
7.0	Rs[a]	4.62±0.03	3.4±0.03	3.42±0.03	1.3±0.07	4.42±0.05	3.6±0.05	3.82±0.03	1.0±0.04	6.7±0.06	6.7±0.02	6.69±0.05	5.63±0.05	53.3	60.0	66.7	66.7
	TC[b]	6.93±0.03	5.7±0.02	5.77±0.03	4.5±0.05	4.65±0.04	5.6±0.02	5.51±0.03	4.5±0.06	8.6±0.12	8.7±0.03	8.60±0.02	6.44±0.03				
8.0	Rs[a]	3.89±0.02	2.5±0.14	2.68±0.04	0.00	4.16±0.35	2.2±0.05	2.32±0.07	0.00	5.6±0.04	4.8±0.05	4.36±0.01	1.48±0.08	0.0	0.0	0.0	0.0
	TC[b]	6.42±0.19	4.7±0.10	2.69±0.04	2.4±0.12	6.43±0.02	4.8±0.05	4.22±0.07	2.9±0.00	8.5±0.10	8.4±0.14	8.45±0.05	6.13±0.01				

[a]Rs-08-17 isolate; [b]Total bacterial count ; [c]A total of 5 eggplants per pot were maintained in two replications for monitoring wilt incidence

Initial Inoculum: 6 log CFU mL^{-1}; Incubation period: 7, 14, 21 and 28 days; pH values (5.0, 6.0, 7.0, 8.0); DAI- Days after inoculation; Culturable cells were enumerated at regular intervals by dilution plating on SMSA medium

The data are presented as the means of three replicates each containing three subsamples; typical results are shown; ± Standard error

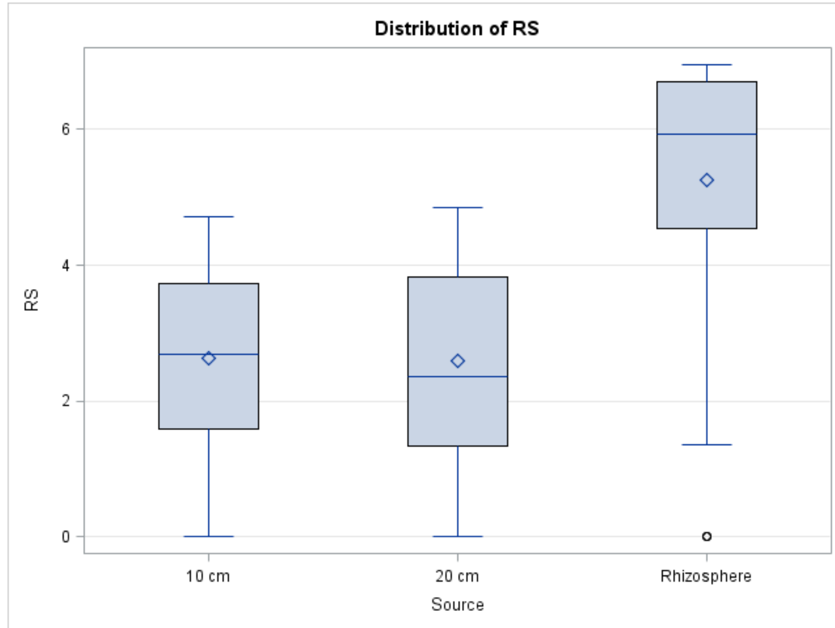
* Pathogen population in soil and rhizosphere region was statistically significant at different pH treatments and incubation time ($P < 0.0001$); R^2 = 0.9

When the total bacterial count was evaluated of the soil it was observed that the population was highest at pH 6.0 and 7.0 at 0-10 cm and 10-20 cm depth (Fig. 4.11 d, e) respectively and the population drastically decreased at pH 5.0 and 8.0. Initially 6 log CFU mL^{-1} of *R. solanacearum* inoculum was introduced into the soil environment. Within 7 days of inoculation there was a significant decrease of 1 log CFU g^{-1} of population at 0-20 cm soil depth (Table 4.5). This could have happened as the direct heat on the soil surface would have killed the bacterium or as the *R. solanacearum* must have migrated inside the soil in search of host. Similar case was reported by Graham and Lloyd (1979), wherein the *R. solanacearum* migrated 75 cm of depth inside the soil. Kinyua *et al.* (1998) also showed that the *R. solanacearum* survives at soil depths of 1m or more, where microbial competition is low. A general decrease of the *R. solanacearum* population in soil has been reported by several authors (Granada and Sequeira, 1983; Quirnio and Chan, 1979; Devi *et al.*, 1981), indicating that *R. solanacearum* does not survive well in non rhizosphere soil. Total bacterial population was very high at pH 6.0, 7.0 and 8.0 in the rhizosphere region of the plant (Fig. 4.11 f) but the bacterial population decreased drastically after 14 days at pH 5.0. Earlier it has been proved that a deviation of 1.5 pH units results in reduction of bacterial growth and activity (Fernandez-Calvino and Baath, 2010). *R. solanacearum* multiplied at a faster rate in the rhizosphere region at pH 6.0 and 7.0. This could be as a result of exudation of certain chemicals or chemo attractants from the roots of eggplant. This type of finding has been also reported by Moffett and Wood (1984).

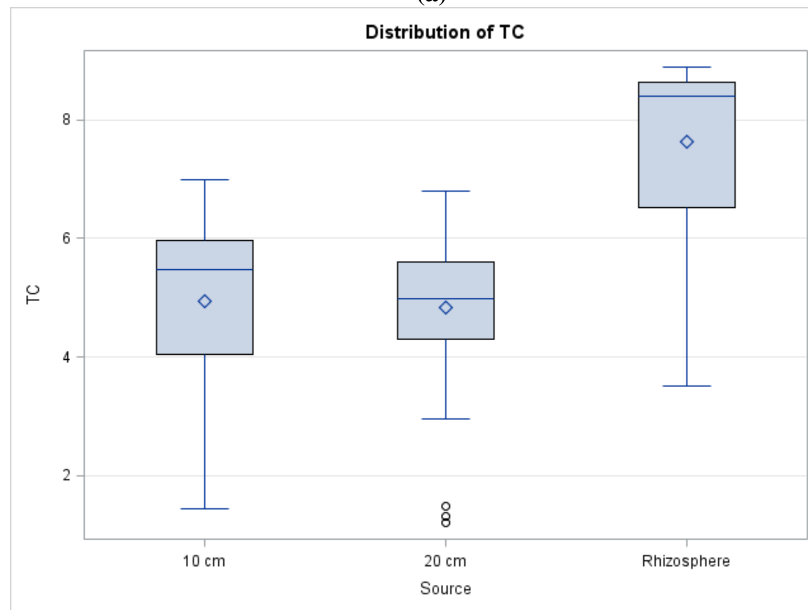
Incidence of wilt was recorded at pH 6.0 and 7.0 on the 7th day post inoculation. Higher number of plants (73%) wilted at pH 6.0 after 21 days of inoculation and at pH 7.0 after

28 days of inoculation (Table 4.5). An increase or decrease in pH reduced wilt in tobacco, tomato and eggplant in fine sandy soils (Kelman, 1953; Michel and Mew, 1998). This study has also witnessed a similar finding at acidic pH (pH 5.0) and alkaline pH (pH 8.0) where in no wilt was observed.

The distribution of the Rs-08-17 and the total bacteria in the soil is depicted in figure 4.12 (a, b). The bacterial population was highest in the rhizosphere (5-6 log CFU g^{-1} in Rs-08-17; 6-8 log CFU g^{-1} in total soil bacteria). Apart from the *R. solanacearum* other bacterial communities too have been in greater numbers in this region when the soil was analyzed. Similar studies on the influence of many soil factors have been studied earlier (Lupwayi *et al.*, 1998; Mahaffee and Klopper, 1997).



(a)



(b)

Fig. 4.12 Distribution range of log transformed (CFU + 1) of (a) *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate and (b) total bacterial population per g dry soil at two soil depths (0-10 cm; 10-20 cm) and rhizosphere region of the eggplant at various soil pH values (5.0, 6.0, 7.0 and 8.0); RS- Population of *R. solanacearum in vivo*; TC- Total bacterial count *in vivo*

4.3.6. Effect of moisture on the survival of Rs-08-17 *in vivo*

The effect of soil moisture level, expressed as a percentage of MHC on Rs-08-17 survival was assessed in glasshouse soils kept at 30°C. There was no significant difference in the population of Rs-08-17 at 0-10 cm and 10-20 cm depth of soil (Fig. 4.13 a, b). At 0-25 % and 26-50% MHC of soil, the population of *R. solanacearum* Rs-08-17 was higher in the soil and in the rhizosphere region of the host as compared to the other MHC treatments. At 51-75 % MHC of soil, *R. solanacearum* survival was observed up to 21 days of inoculation in the soil. At 76-100 % MHC of soil, *R. solanacearum* failed to survive after 14 days of inoculation in the soil (Fig. 4.13 a, b). Overall observations showed that Rs-08-17 could survive at 0-25% and 26-50% MHC values even after 28 days post inoculation in the soil (Table 4.6).

Rhizosphere population was highest at 0-25% and 26-50% MHC treatments (Fig. 4.13 c). *R. solanacearum* population gradually decreased at 51-75% up to 28 days in the rhizosphere region. At 76-100 % MHC of soil, *R. solanacearum* failed to survive after 14 days of inoculation in the rhizosphere region (Fig. 4.13 c).

The influence of total bacteria present in the soil on the survival of *R. solanacearum* was also studied. Results indicated that the microbial count at 0-10 and 10-20 cm depth of soil remained unchanged (Fig. 4.13 d, e). The total soil bacteria remained in high numbers at the rhizosphere region (Fig. 4.13 f). The total bacteria were highest at 0-25% and 26-50%. It slightly declined within 7 to 14 days of inoculation and remained constant thereafter.

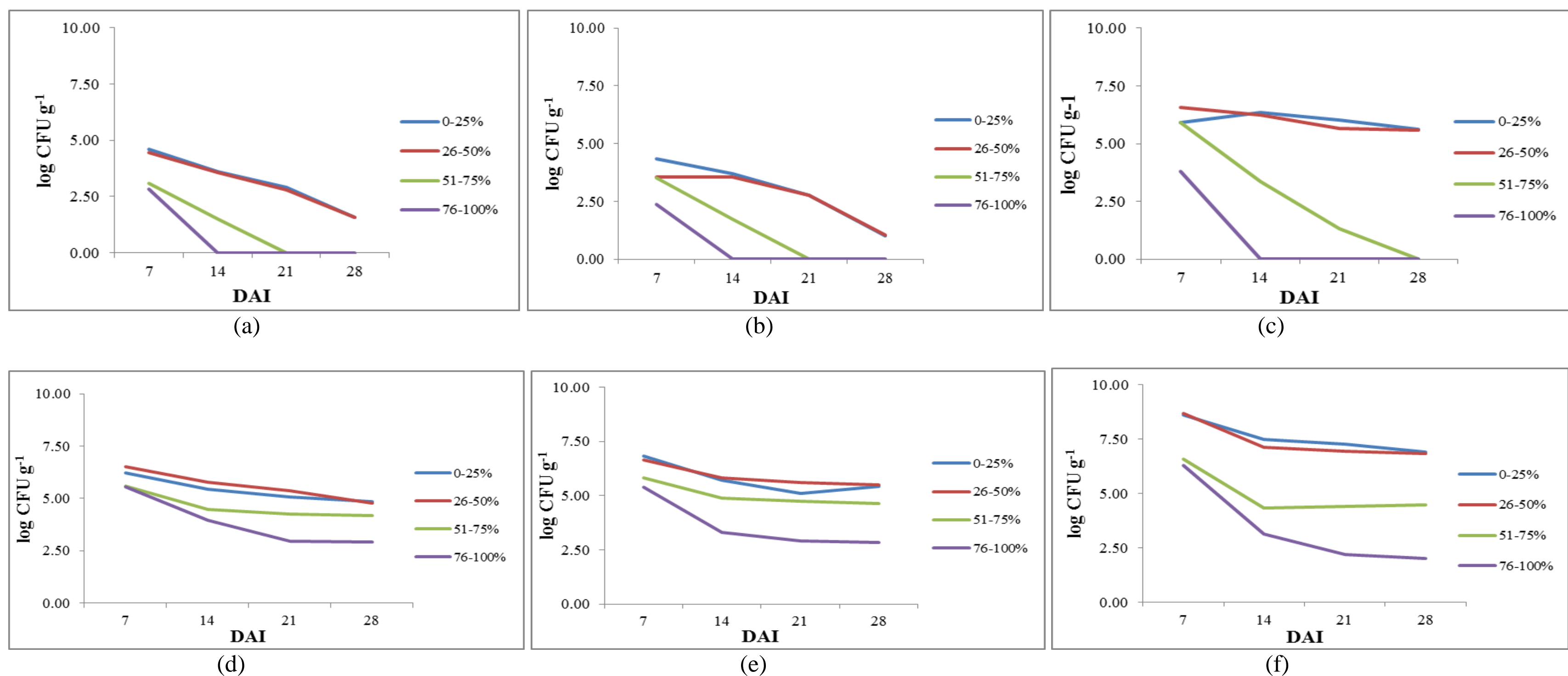


Fig. 4.13 Survival of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate and soil bacteria under different MHC treatments. Soil samples removed from (a, d) 0-10 cm depth; (b, e) 10-20 cm depth and (c, f) rhizosphere region of eggplant. The population of the bacteria was calculated on day 7, day 14, day 21 and day 28 by plating on SMSA (for Rs-08-17 population) and KB medium (for total bacterial population). Data shown reflect the average of three experiments. The mean values of the population were transformed into log CFU g^{-1}

a, b, c- Rs-08-17 Population *in vivo*; d, e, f- Total bacterial population *in vivo*

Wilt incidence was recorded at 0-25%, 26-50% and 51-75% MHC treatments within 7 days post inoculation. 73% of plants wilted at 0-25% and 26-50% MHC treatments after 21 days of inoculation. At 51-75% MHC, only 6.7% eggplants wilted in 7 days after which no wilting took place (Table 4.6).

Table 4.6 Population of *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate and total soil bacteria at varying MHC values in soil and in the rhizosphere region of host.

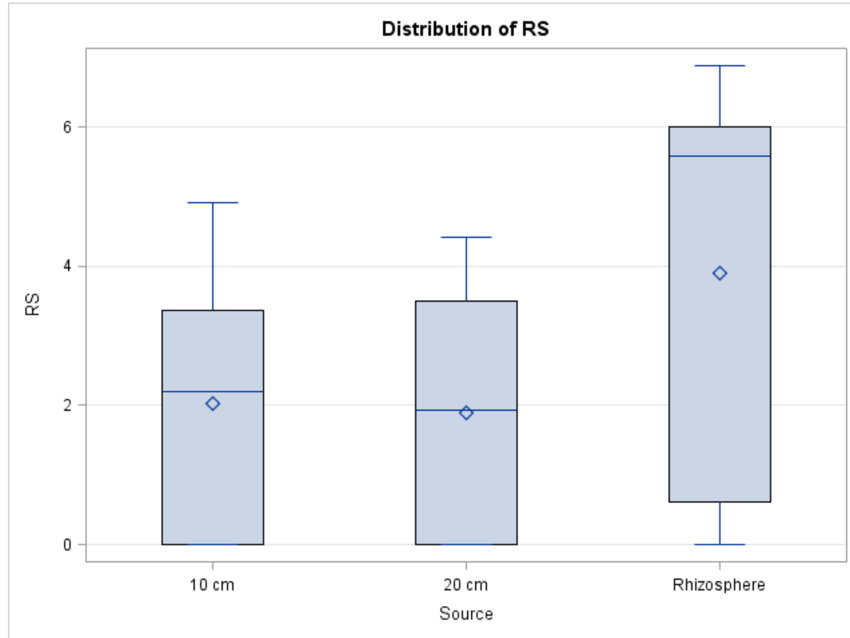
MHC* (%)	Source	Sampling site*												Wilt incidence[c] (%)			
		0-10 Cm depth				0-20 Cm depth				Rhizosphere region							
		7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI	7DAI	14DAI	21DAI	28DAI
	Rs[a]	4.6±0.18	3.61±0.06	2.9±0.04	1.6±0.05	4.4±0.04	3.69±0.09	2.76±0.02	1.0±0.01	5.9±0.04	6.35±0.03	6.0±0.03	5.6±0.02	46	60	73	73
0-25	TC[b]	6.2±0.04	5.45±0.09	5.0±0.01	4.8±0.03	6.8±0.04	5.72±0.08	5.11±0.32	5.4±0.06	8.6±0.19	7.46±0.07	7.3±0.04	6.9±0.01				
	Rs[a]	4.4±0.03	3.59±0.02	2.8±0.0	1.57±0.05	3.6±0.06	3.56±0.08	2.76±0.04	1.0±0.03	6.6±0.27	6.24±0.06	5.6±0.29	5.6±0.02	53	66	73	73.3
26-50	TC[b]	6.5±0.22	5.78±0.01	5.3±0.01	4.76±0.03	6.6±0.08	5.81±0.03	5.59±0.05	5.5±0.07	8.7±0.10	7.13±0.07	6.9±0.04	6.8±0.02				
	Rs[a]	3.1±0.06	1.52±0.09	0.00	0.00	3.5±0.12	1.74±0.10	0.00	0.00	5.9±0.02	3.36±0.14	1.3±0.05	0.00	6.7	6.7	6.7	6.7
51-75	TC[b]	5.6±0.15	4.47±0.06	4.3±0.02	4.2±0.09	5.8±0.03	4.9±0.02	4.75±0.03	4.6±0.02	6.6±0.26	4.34±0.14	4.4±0.06	4.5±0.01				
	Rs[a]	2.8±0.05	0.00	0.00	0.00	2.4±0.19	0.00	0.00	0.00	3.8±0.10	0.00	0.00	0.00	0.0	0.0	0.0	0.0
76-100	TC[b]	5.6±0.12	3.93±0.03	2.9±0.01	2.9±0.02	5.4±0.07	3.3±0.04	2.91±0.03	2.8±0.01	6.3±0.03	3.13±0.06	2.2±0.03	2±0.01				

[a]Rs-08-17 isolate; [b]Total bacterial count ; [c]A total of 5 eggplants per pot were maintained in two replications for monitoring wilt incidence

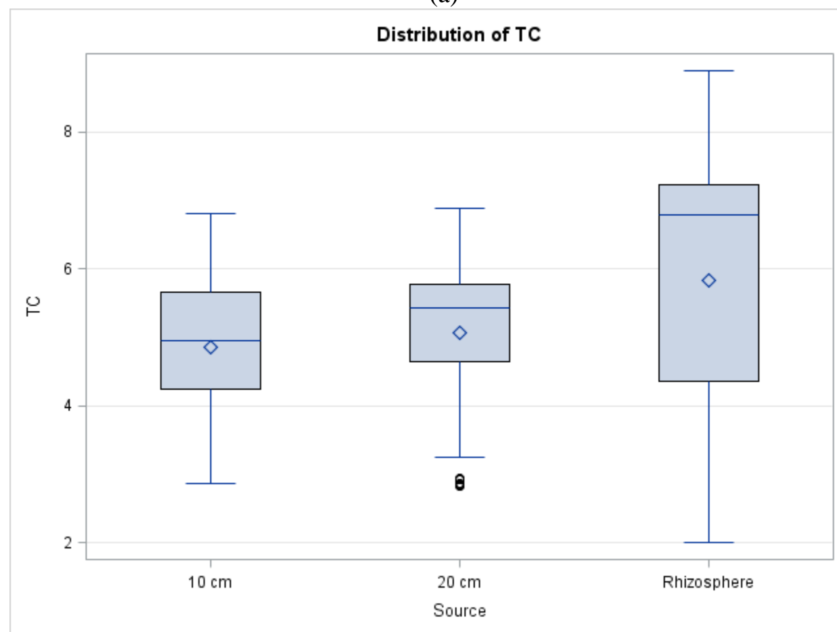
Initial Inoculum: 6 log CFU mL^{-1}; Incubation period: 7, 14, 21 and 28 days; MHC values in % (0-25, 26-50, 51-75, 76-100); DAI- Days after inoculation; Culturable cells were enumerated at regular intervals by dilution plating on SMSA medium

The data are presented as the means of three replicates each containing three subsamples; typical results are shown; ± Standard error

* Pathogen population in soil and rhizosphere region was statistically significant at different MHC treatments and incubation time ($P < 0.0001$); $R^2 = 0.8$



(a)



(b)

Fig. 4.14 Distribution range of log transformed (CFU + 1) of (a) *R. solanacearum* Race 1 biovar 3 Rs-08-17 isolate and (b) total bacterial population per g dry soil at two soil depths (0-10 cm; 10-20 cm) and rhizosphere region of the eggplant at various MHC values (0-25%, 26-50%, 51-75% and 76-100%); RS- Population of *R. solanacearum in vivo*; TC- Total bacterial count *in vivo*

Da Silva *et al.* (2003) have also found that soil moisture is one of the factors that determine the bacterial communities in the rhizosphere region. Keshwal *et al.* (2000) reported that Sandy loam soil with 34.6% water holding capacity (WHC) harbored a minimum population of *R. solanacearum* PST4 inciting 32.2% wilt in tomato crop. However, in this study the *R. solanacearum* survival was highest at 0-25% and 26-50% MHC treatment. But it failed to survive at 76-100% MHC. This may be due to the establishment of anaerobic conditions thereby declining the *R. solanacearum* population and eventually the death. However, Pereira and Normando (1993) reported that the survival of *R. solanacearum* Race 2 was enhanced by high water content in soil. Another report stated that poor drainage and consequently high soil moisture levels are responsible for high disease incidence (Mickail *et al*., 1985).The results in this study are in conformity with the reports presented by Champoiseau *et al.* (2009) that *R. solanacearum R. solanacearum* survives in wet but well-drained soils. However, soil desiccation and flooding has a negative effect on the *R. solanacearum* growth (Buddenhagen and Kelman, 1964; Nesmith and Jenkins, 1985). Studies have also proved that severe drought negatively affects *R. solanacearum* survival (Van Elsas *et al.*, 2000).

The survival of *R. solanacearum* in soil has received considerable attention, and the survival period is influenced by a range of environmental factors. The aggressiveness of this *R. solanacearum* is affected mainly by temperature and moisture; high temperature, and high soil moisture promote survival, reproduction, infectivity, and spread of the bacterium, and hence disease development (Harris, 1976; Martin and French, 1985). Factors affecting survival in soil may, thus, be intertwined, and the responses of the bacterial populations studied will depend on the relative impact of each factor.

From these results, it can be concluded that Rs-08-17 grew favorably at pH 6.0 when MHC was 26-50% at 30°C and could wilt 60-70% of the host within 7-14 days of inoculation. However, Rs-08-17 was inhibited by high salt concentrations, extreme temperatures (below 20°C, above 37°C), extreme pH (below 6, above 8) and very high level of moisture content in soil. In spite of the fact that certain plants enhance and help the *R. solanacearum* persistence, direct survival in the soil or in soil-associated water at varying soil temperatures and pH possibly cannot be ruled out as an important factor that determines the fate of *R. solanacearum* following field infestation.

Chapter V

Effects of botanicals on the inhibition of *R. solanacearum*

5.1. Introduction

R. solanacearum is regarded as one of the most important bacterial phytopathogen causing BW mainly in the solanaceous vegetables in India (Singh *et al.*, 1997). The disease is known to cause huge losses on a variety of economically important crops worldwide. The pathogen affects crops such as banana, tobacco, peanut, tomato, eggplant, pepper and potato. Therefore approaches to control BW such as cultivation of resistant varieties (Gopalakrishnan *et al.*, 2005), crop rotation (Lemaga *et al.*, 2001), foliar application of copper pesticides (Ji *et al.*, 2005), selection of healthy planting material (Guo *et al.*, 2004), organic soil amendments (Getachew *et al.*, 2011) and application of biocontrol agents (Ramesh *et al.*, 2009; Xue *et al.*, 2009) have been reported that have shown limited success. Biological control of the BW is a very promising approach to combat the disease (Guo *et al.*, 2004; Xue *et al.*, 2009; Thanh *et al.*, 2009) since its environmental friendly and therefore an acceptable practice compared to others. Despite the efforts of several research teams, the efficiency of current strategies for the management of BW is still limited. However, BW management using a single conventional method is not effective and therefore alternative practices to manage the disease were reported (Alemu *et al.*, 2013) and hence the use of plant-derived compounds to control bacterial diseases has been the subject of increasing interest (Balestra *et al.*, 2009; Gurjar *et al.*, 2012).

Plants represent an untapped source of novel compounds that might serve as leads for development of novel drugs (Cowan, 1999) and promising chemotherapeutants (Dorman and Deans, 2000). Plants are rich sources of bioactive compounds, such as terpenoids, alkaloids, flavonoids, tannins and polyacetylenes (Harborne, 1993). Plant extracts and secondary metabolites from plants have been used by many researchers in disease control over synthetic bactericides (Abera *et al.*, 2011). Thymol, a volatile compound from plants when used as pre-plant soil fumigation was found to reduce bacterial wilt incidence on tomato (Ji *et al.*, 2005). Deberdt *et al.* (2012) have reported the *in vitro* inhibition of *R. solanacearum* phylotype IIB/4NPB by *Allium fistulosum* extracts. The antibacterial effect of crude medicinal plant extract of *Ocimum gratisimum*, *Brassica oleracae* and *Ipomoea batatas* on *R. solanacearum* were also reported by Wagura *et al.* (2011). Extracts of *Myrtus communis*, *Lantana camara*, *Cassia sp.* (Moussa *et al.*, 2010) and *Morinda citrifolia* (Sunder *et al.*, 2011) were found to be very effective against *R. solanacearum* inhibition. Inhibition of *R. solanacearum* by plant extracts have been reported recently (Lemos, *et al.*, 2005; Lopez *et al.*, 2005; Larkin and Griffins, 2007; Walters, 2009). Sangoyomi *et al.* (2011) reported that aqueous extracts of medicinal plants failed to inhibit the pathogen growth and therefore it would be wise to study the effects of different solvent extracts other than water in repressing the growth of *R. solanacearum.*

This study was undertaken with the aim to evaluate the antibacterial activity of solvent extracts from thirty two different plants based on the availability but not limited to weedy and invasive alien species against *R. solanacearum*. The phytochemical properties of the promising plant extracts were also carried out.

5.2. Materials and methods

5.2.1. Collection of Plant material

Thirty two plant species were selected for the study and their details are given in Table 5 A.

Table 5 A. Details of the plant species used under the study

No.	Plant species	Plant part used
1	*Murraya koenigii*	Leaves, root
2	*Eupatorium odorata*	Leaves
3	*Thuja occidentalis*	Leaves
4	*Azadirachta indica*	Leaves
5	*Ocimum tenuiflorum*	Leaves
6	*Adhathoda vasica*	Leaves
7	*Garcinia indica*	Leaves
8	*Moringa oleifera*	Leaves
9	*Saraca asoca*	Leaves
10	*Hibiscus sp.*	Leaves
11	*Jatropha curcas*	Leaves
12	*Aegle marmelos*	Leaves
13	*Carica papaya*	Leaves
14	*Anacardium occidentalis*	Leaves
15	*Ocimum basilicum*	Leaves
16	*Cinnamomum zevlancium*	Leaves
17	*Myristica fragrans*	Leaves
18	*Glyricidia sp.*	Leaves
19	*Zingiber officinale*	Rhizome
20	*Psidium guajava*	Leaves
21	*Lawsonia intermis*	Leaves
22	*Averrhoe carambola*	Leaves
23	*Alstonia sp.*	Leaves
24	*Canavalia gladiata*	Leaves
25	*Acacia auriculiformis*	Leaves
26	*Ricinus sp.*	Leaves
27	*Physalis weed*	Whole plant
28	*Mimosa pudica*	Leaves
29	*Calotropis gigantea*	Leaves
30	*Tamarindus indica*	Leaves
31	*Boerhavia diffusa*	Whole plant
32	*Cybopogon flexuosus*	Leaves

These species were commonly grown and readily available in Goa. The plant parts such as their leaves, root and whole plant were collected separately and washed thoroughly with normal tap water twice. Later the samples were washed using sterile distilled water. The plant materials were dried at room temperature under shade for 3 to 4 days until completely dried. Dried material was grounded using a grinding machine into fine powder and the powder was stored in polythene bags at 4°C until further extraction.

5.2.1.1. Sample Preparation and extraction

Extraction of the powdered plant material (leaf, root, whole plant part) was done with 8 different organic solvents (80% ethanol, methanol, chloroform, 80% ethyl acetate, acetone, dichloromethane, diethyl ether and hexane) as per the protocol of Nduagu *et al.* (2008) with slight modifications. 5g of the dry powder of the plant sample was initially soaked in 25 ml of organic solvents to get 20% (w/v) extract in a 100mL Erlenmeyer flask. The flask was covered with paper and later kept on shaker for 48 h at 125 rpm. The infusions were filtered after 48 h using Whatman No. 1 filter paper. The collected filtrate was then concentrated in vacuum and dried at 40°C in a hot air oven. After the solvent evaporation, the residue was dissolved in 500µL of the respective solvent and stored in airtight sterile tubes in a refrigerator at 4°C until further use. The crude extract thus obtained was directly used in the bioassay.

5.2.2. *In vitro* antibacterial activity assay of plant extracts against *R. solanacearum*

Rs-08-17 isolate being the most virulent of all the isolates under this study was used in this bioassay. Antibacterial activity of the plant extract against *R. solanacearum* isolate was carried using agar diffusion method (Shrisha *et al.*, 2011). *R. solanacearum* Rs-08-17 was grown overnight and the bacterial population was adjusted to 6 log CFU mL^{-1}. 25µL

of the culture was added to 100mL of molten CPG medium and poured into sterile Petri plates and allowed to solidify. 5 mm diameter wells (4 in number) were punctured in the agar medium per plate using sterile cork borer. 35µL of crude extract from each sample was added in 3 wells per plate and plain solvent to the 4th well. Negative control was maintained by adding CPG broth in a plate seeded with the bacterium. Streptomycin (50 $\mu g\ \mu L^{-1}$) was used as positive control in the bioassay. Plates were incubated at $28 \pm 2^{\circ}C$ for 48 to 72 h. Zone of inhibition of plant extract was measured after subtracting zone of inhibition if any in the control by the corresponding plain solvent. The experiment was conducted in a completely randomized design and three replicates were maintained per treatment. Activity index (AI) was also estimated using the formula described by Shahidi (2004) and expressed as %. AI= 100 x diameters of inhibition zone of extract ÷ diameters of inhibition zone of the standard antibiotic.

5.2.2.1. Determination of the shelf life of the plant extract

After careful observation of the results of the above mentioned assay a few plants (*P. guajava*, *T. indica*, *M. pudica*, *G. indica*, *A. auriculiformis*, *A. occidentalis and C. zevlancium*) were shortlisted based on the most promising results acquired under the invitro antibacterial assay. The stability and shelf life of the plant extracts was measured using a year old stored crude plant extract by re-testing their *in vitro* antibacterial activity as mentioned in section 5.2.2. The observations were compared with the previous screening reports.

5.2.3. Phytochemical analysis

G. indica and *T. indica* (ethanol extracts) showed consistent antibacterial activity against the pathogen same like a year before. Therefore these extracts were selected for

preliminary qualitative phytochemical analysis for the presence of flavonoids, alkaloids, tannins, terpenoids and saponins. A stock solution of the plant product was prepared by dissolving 1g of crude extract in 100 mL of respective solvents to be used for further phytochemical screening following the standard methodology described elsewhere.

Test for tannins: 2 mL each of extracts were taken and 10 mL of distilled water was added to it. This solution was boiled for 10 min in a test tube. Later a few drops of 0.1% ferric chloride were added to each test tube and incubated for 10 min for the development of brownish green or a blue black color that indicated the presence of tannin (Okwu, 2005).

Test for saponins: 5 mL each of the extracts were taken and to that equal amount of distilled water was added in a test tube and was shaken for 5 min. The mixture was boiled in the water bath for 5-10 min. Frothing during warming of the solution indicated the presence of saponins (Trease and Evans, 1989).

Test for flavonoids: 3 mL of the extracts were taken and 1 mL of 10% NaOH was added to it. A yellow coloration showed the presence of flavonoids (Okwu, 2005).

Test for terpenoids: 500 mg of each plant extract was taken in separate test tubes and 2 mL of chloroform was added. Later concentrated H_2SO_4 was carefully added by the side of the test tube and observed for formation of a reddish brown color interface layer indicating the presence of terpenoids (Mehta *et al.*, 2013).

Test for alkaloids: 1 mL of the extract was taken and 2 drops of Mayer's reagent (Appendix B.2) was added. Formation of a creamy precipitate indicated the presence of alkaloids (Sofowora, 1993).

5.2.3.1. Solvent-solvent partitioning method

This method works on the principle of distributing the components in the mixture depending on the solvent polarity. The protocol devised by Tefera *et al.* (2010) was followed with slight modifications. Ethanol extract (15g) of the plant was suspended in 50 mL of distilled water and mixed thoroughly. To this mixture 50 mL of chloroform was added each time and shaken until the suspension became colorless and allowed to be partitioned in to chloroform-aqueous layer. The chloroform fraction was collected, filtered and evaporated in a rotary evaporator and labeled as Fraction one (F-I). To the remaining aqueous fraction, 25 mL of n-butanol was added, shaken and allowed to be separated into n-butanol-aqueous phase. The n-butanol layer was filtered, concentrated and evaporated to dryness on water bath ($40^{\circ}C$) which was labeled as Fraction two (F-II). The remaining aqueous fraction was now labeled as Fraction three (F-III). All the three fractions were dried in the vacuum evaporator and re-dissolved in 500µL of their respective organic solvents. Antibacterial activity of these fractions was tested against *R. solanacearum* Rs-08-17 according to the method described in the *in vitro* antibacterial activity assay mentioned in section 5.2.2. Zones of inhibition were determined and the fractions effective against *R. solanacearum* were further analyzed using GC-MS.

5.2.4. Gas chromatography-mass spectrometer (GC-MS) analysis

Fraction III from *G. indica* and *T. indica* proved to be highly inhibitory against the pathogen. Therefore GC-MS technique was used to identify the components present in the extract (F-III from solvent-solvent partition). GC-MS analysis of the two fraction (F-III) was carried out at Sophisticated Analytical Instrument Facility, Indian Institute of Technology- Bombay, Powai (Mumbai) using an Agilent Technologies 7890A GC

systems interfaced to a Mass Spectrometer Jeol The AccuTOF GCV JMS-T100GCV with Hewlett-Packard HP-5MS (30m x 0.25mm x 0.25μm) column. For GC-MS detection, an electron ionization energy system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min. 2μl of the Fraction-III was used as injection volume at a split ratio of 20:1. Injector temperature of 250°C and Ion-source temperature of 200°C were set. The oven temperature was set as follows: 80° C (isothermal for 1 min) with an increase of 8°C min^{-1} to 200°C, followed by 8°C min^{-1} to 250°C with 5 min isothermal at 280°C. GC was run for 36 min. Interpretation on mass spectrum GC-MS was done using the SAIF-IIT database. The spectrum of the unknown component was compared with the spectrum of the known components available in the respective library. From the database, name, molecular weight and structure of the components of the F-III were ascertained.

5.3. Results and Discussion

5.3.2. *In vitro* antibacterial activity assay of plant extracts against *R. solanacearum*

A total of 264 crude extracts were prepared from 32 plant species using 8 organic solvents and screened for their antibacterial activity against *R. solanacearum* Rs-08-17. *In vitro* studies on antibacterial activities of aqueous, ethanol, methanol, chloroform, ethyl acetate, acetone, dichloromethane, diethyl ether and hexane extracts against the growth of *R. solanacearum* Rs-08-17 were conducted and the results obtained were significantly different (Table. 5.1).

123 extracts showed variable inhibitory activity against the pathogen. *In vitro* antibacterial test results showed that most of the plant extracts exhibited antibacterial activity by developing distinct inhibitory zone around agar wells against *R. solanacearum* (Fig. 5.1).

Table 5.1 Antibacterial activity of plant extracts in different organic solvents against *R. solanacearum* Race 1 biovar 3 Rs-08-17

No.	**Plant species**	**Plant part used**	**Inhibition zone of plant extracts against *R. solanacearum* growth (diameter in mm)***							
			80% Ethanol	Methanol	Acetone	80% Ethyl Acetate	DCM	DEE	Chloroform#	Hexane
1	*Murraya koenigii*	Leaves	10.6±0.27	-	-	-	-	-	10.3±0.27	-
		Root	9.0±0.0	-	-	-	-	6.0±0.0	6.3±0.27	-
2	*Eupatorium odorata*	Leaves	8.3±0.54	10.0±0.0	-	-	-	-	-	-
3	*Thuja occidentalis*	Leaves	8.0±0.0	12.6±0.27	11.3±0.27	15.0±0.47	-	-	-	-
4	*Azadirachta indica*	Leaves	12.6±0.54	-	-	9.0±0.0	-	-	8.3±0.27	-
5	*Ocimum tenuiflorum*	Leaves	15.0±0.0	11.0±0.0	-	13.0±0.0	-	-	9.0±0.0	-
6	*Adhathoda vasica*	Leaves	14.6±0.27	-	6.0±0.0	-	7.0±0.0	-	9.0±0.47	-
7	*Garcinia indica*	Leaves	25.3±0.27	30.0±0.0	20.6±0.54	9.3±0.27	-	9.6±0.27	8.0±0.0	-
8	*Moringa oleifera*	Leaves	15.0±0.0	10.3±0.0	8.0±0.0	14.0±0.47	8.3±0.27	12.0±0.0	8.6±0.0	-
9	*Saraca asoca*	Leaves	12.6±0.54	-	9.3±0.27	9.6±0.27	8.0±0.0	-	7.0±0.0	-
10	*Hibiscus sp.*	Leaves	12.0±0.0	7.6±0.27	-	-	7.6±0.27	6.0±0.0	-	-
11	*Jatropha curcas*	Leaves	9.2±0.27	7.7±0.27	-	-	-	-	9.5±0.0	-
12	*Aegle marmelos*	Leaves	12.6±0.27	10.6±0.27	12.6±0.27	16.3±0.72	11.3±0.27	-	8.0±0.0	-
13	*Carica papaya*	Leaves	12.0±0.0	14.0±0.47	10.3±0.27	8.0±0.47	-	-	6.3±0.54	-
14	*Anacardium occidentalis*	Leaves	20.3±0.27	24.6±0.27	-	9.6±0.27	-	10.5±0.47	9.0±0.0	-
15	*Ocimum basilicum*	Leaves	12.0±0.47	8.0±0.0	8.6±0.27	11.0±0.0	9.0±0.0	-	13.0±0.47	-
17	*Myristica fragrans*	Leaves	9.0±0.27	-	-	-	7.3±0.27	-	-	-
18	*Glyricidia sp.*	Leaves	12.0±0.0	9.6±0.27	-	12.6±0.27	-	-	8.0±0.0	-
19	*Zingiber officinale*	Rhizome	12.6±0.54	12.0±0.47	-	12.3±0.27	-	-	9.6±0.27	-
20	*Psidium guajava*	Leaves	31.6±0.27	28.0±0.47	28.3±0.54	12.0±0.0	-	-	14.0±0.0	-
21	*Lawsonia intermis*	Leaves	14.0±0.0	20.6±0.98	17.6±0.27	17.3±0.27	8.0±0.0	14.0±0.0	6.0±0.47	-
22	*Averrhoe carambola*	Leaves	15.0±0.0	11.6±0.0	9.0±0.0	-	-	-	-	-
23	*Alstonia sp.*	Leaves	14.0±0.0	11.3±0.27	-	11.0±0.0	6.3±0.27	-	6.0±0.0	-
24	*Canavalia gladiata*	Leaves	14.3±0.27	10.0±0.0	16.0±0.0	11.6±0.54	-	-	-	-
25	*Acacia auriculiformis*	Leaves	25.0±0.0	9.0±0.0	23.6±0.0	11.3±0.0	9.3±0.0	-	10.0±0.0	-
26	*Ricinus sp.*	Leaves	11.0±0.0	12.6±0.27	-	10.0±0.0	-	-	9.6±0.27	-
27	*Physalis weed*	WP	8.3±0.0	17.6±0.0	9.0±0.0	9.6±0.0	-	-	11.3±0.0	-

No.	Plant species	Plant part used	Inhibition zone of plant extracts against *R. solanacearum* growth (diameter in mm)*							
			80% Ethanol	Methanol	Acetone	80% Ethyl Acetate	DCM	DEE	Chloroform#	Hexane
28	*Mimosa pudica*	Leaves	20.6±0.0	13.6±0.54	6.0±0.0	14.3±0.0	9.0±0.0	-	8.0±0.0	-
29	*Calotropis gigantea*	Leaves	20.0±0.0	15.0±0.0	14.0±0.0	15.3±0.47	7.0±0.0	-	8.0±0.0	-
30	*Tamarindus indica*	Leaves	23.0±0.27	21.6±0.27	30.6±0.27	13.6±0.54	10.0±0.0	-	9.0±0.0	-
31	*Boerhavia diffusa*	WP	15.0±0.0	16.6±0.0	12.0±0.0	30.0±0.0	8.9±0.0	-	-	-
32	*Cybopogon flexuosus*	Leaves	13.0±0.0	-	11.6±0.0	31.6±0.54	10.0±0.0	-	-	-
	Control	-	-	-	-	-	-	-	8.0	-

*The data represents the mean values of three replicates with (±) standard deviation; (-) indicates no inhibition; Streptomycin (50 µg/µL) control recorded 40 mm inhibition zone; Zone of inhibition of plant extract was measured after subtracting zone of inhibition if any by the corresponding solvent; WP-Whole plant; DCM- Dichloro methane; DEE- Diethyl ether; # Chlorofrom solvent control inhibited Rs-08-17 (8.0 mm)

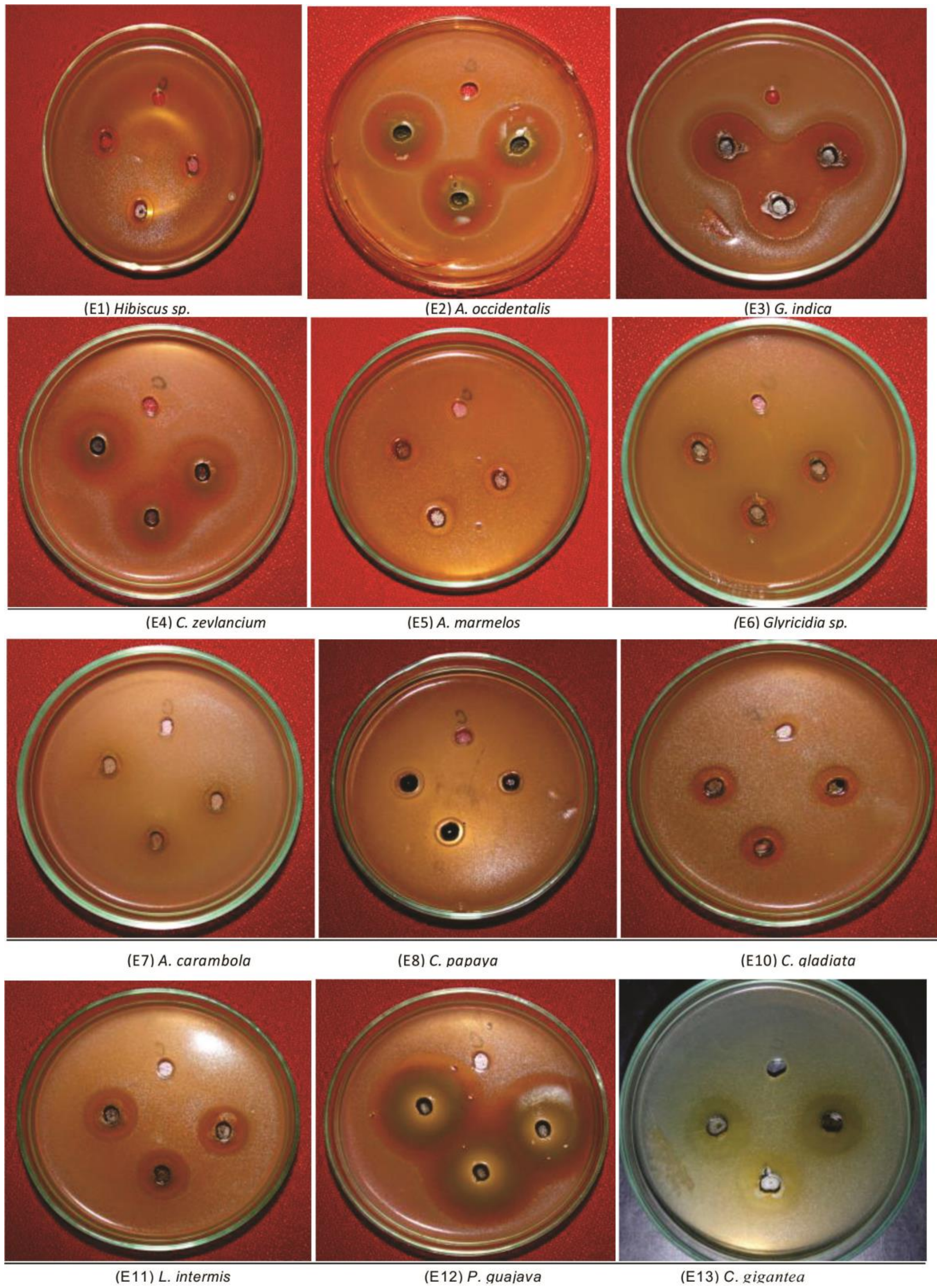


Fig. 5.1 Inhibition of *R. solanacearum* Rs-08-17 by various plant extracts: Plates named E11-E13 are ethanolic extracts

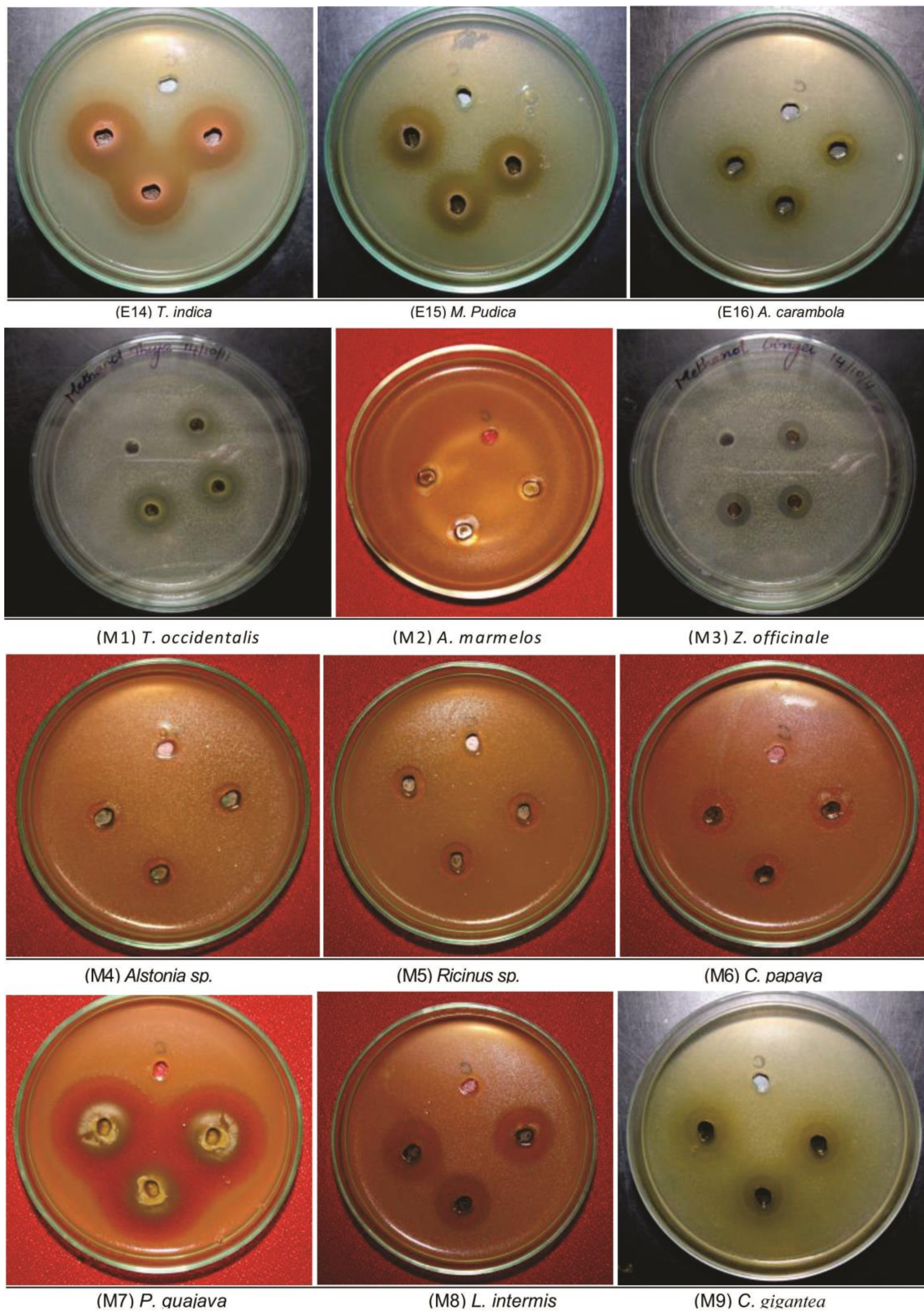


Fig. 5.1 Inhibition of *R. solanacearum* Rs-08-17 by various plant extracts: Plates named E14-E16 are ethanolic extracts; Plates named M4-M9 are methanolic extracts

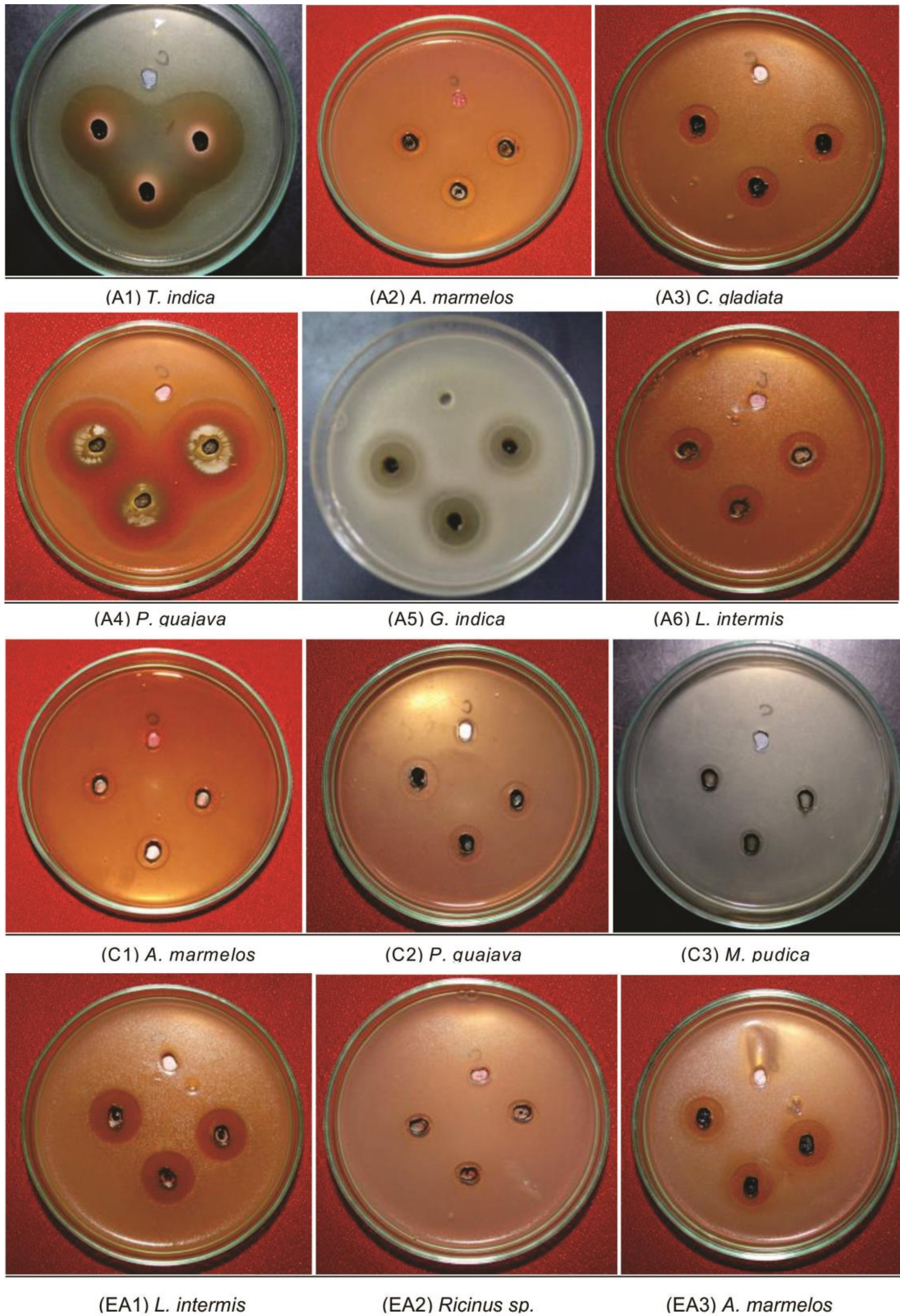


Fig. 5.1 Inhibition of *R. solanacearum* Rs-08-17 by various plant extracts: Plates named A1-A6 are acetone extracts; Plates named EA1-EA3 are ethyl acetate extracts

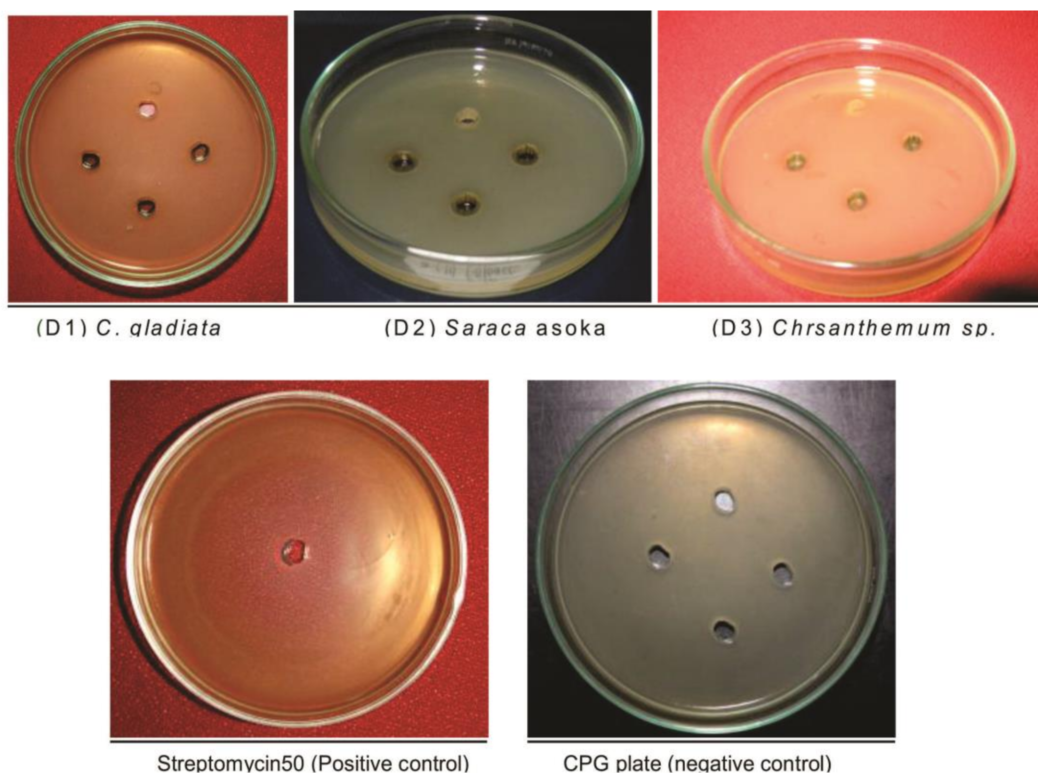


Fig. 5.1 Inhibition of *R. solanacearum* by various plant extracts: Plates named D1-D3 are Diethyl ether extracts. Control plates: Streptomycin50 (Positive control); CPG agar (Negative control).

In this study, plant extracts were prepared in eight different organic solvents and the inhibitory activity of these extracts against Rs-08-17 was in the range of 6.0-31.6 mm (inhibition zone). The *in vitro* inhibitory activity of ethanol extracts of plants against *R. solanacearum* ranged from the inhibition zone of 8.0 mm to 31.6 mm. Ethanol extract of *P. guajava* provided the highest diameter of inhibition zone (31.6 mm) followed by *G. indica* (25.3 mm), *A. auriculiformis* (25.0 mm) and *T. indica* (23.0 mm), *M. pudica* (20.6 mm), *A. occidentalis* (20.3 mm) and *C. gigantea* (20.0 mm). Methanol extract of *G. indica* (30.0 mm) showed the highest inhibitory zone followed by *P. guajava* (28.0 mm), *A. occidentalis* (24.6 mm), *T. indica* (21.6 mm) and *L. intermis* (20.6 mm). Acetone extracts of *T. indica* (30.6 mm) displayed highest diameter of zone of inhibition followed by *P. guajava* (28.3 mm), *A. auriculiformis* (23.6 mm) and *G. indica* (20.6 mm). Highest

inhibitory zone was exhibited by ethyl acetate extracts of *C. flexuosus* (31.6 mm) followed by *B. diffusa* (30.0 mm) and *C. zevlancium* (24.6 mm). With chloroform extract, the highest diameter of inhibition zone was displayed by *P. guajava* (14.0 mm) followed by *O. basilicum* (13.0 mm) that was significantly higher as compared to the other plant species but the plain solvent was found to inhibit the pathogen in the control. Though inhibition zones were also produced by dichloromethane and diethyl ether extracts of some of the plant species, they were in the range of 6.0-14.0 mm. Hexane extract of the plant species were not inhibitory to *R. solanacearum* (Table 5.1). Results from this study indicate that vast group of plants exhibit antibacterial activity, which further proves that these plants are the unexploited sources of antimicrobial compounds.

Chanda and Kaneria (2011) reported that accumulation of bioactive compounds as secondary metabolites in all plant cells, however their concentration is high in leaf tissues among the plant parts (Abreu *et al*., 2003). Therefore leaves of 29 different plant species, root portion of one plant, whole plants of 2 botanicals and rhizome portion of one were used for extraction of effective and potent anti-*Ralstonia* compounds. A recent study reported that antibacterial activity was observed in the organic solvent as compared to water, which indicates that the active compounds responsible for the bactericidal activity are more soluble in the organic solvents (Vinoth *et al*., 2012). Similar finding was also reported by Sangoyomi *et al.* (2011) that aqueous extracts of medicinal plants failed to inhibit *R. solanacearum* and therefore plant extracts extracted from different solvent need to be studied against the pathogen.

The results of the present work indicated that the organic solvent extracts exhibited greater antibacterial activity because the antimicrobial principle were either polar or non-

polar and this is supported by many investigators (Raghavendra *et. al*, 2009; Mohanasundari *et al.*, 2007; Praveen *et al.*, 2011). Recently, the anti-*R. solanacearum* activity of various plant extracts was reported by a number of authors (Owoseni and Sangoyomi, 2014; Wagura *et al*., 2011; Lemos *et al.*, 2005; Lopez *et al.*, 2005; Larkin and Griffins, 2007; Walters, 2009). Subin and Dilna (2012) revealed from their studies that acetone extracts of *B. diffusa* showed better inhibition against *R. solanacearum*. However, it was found that ethyl acetate exhibited a diameter of inhibition zone of 30.0 mm against the pathogen in this study.

Overall observations proved that 33 Ethanol extracts, 26 methanol extracts, 25 ethyl acetate extracts, 19 acetone extracts, 15 dichloromethane extracts and 5 diethyl ether extracts showed zones of inhibition. A total of 20 plant extracts *viz*. 8 ethanol, 5 methanol, 4 acetone and 3 ethyl acetate extracts showed >20 mm size zones of inhibition (Table 5.1).

This study demonstrated that the interactions between the plant extracts and their organic solvents were highly significant. This was proven from the fact that compounds extracted from all the plants using different solvents vary in their efficiency in inhibiting *R. solanacearum* growth. This may be dependent on the solubility properties of the active compounds or due to the presence of inhibitors in the organic solvents used. The findings of this study are in line with that which is reported by Marjorie (1999). Though many mechanisms were suggested for the inhibition, it is likely that these extracts inhibit the multiplication of bacterial cells as evidenced by the lack of growth.

Alam *et al.* (2009) has reported methanol as the most potent solvent for extracting phytochemicals from plant materials. *In vitro* antibacterial assay against Rs-08-17

indicated that highest number of ethanol and methanol extracts of all the samples showed much better antibacterial activity in contrast to other solvents with a few exceptions. This could be due to the fact that these alcoholic solvents possess higher capacity to dissolve more organic and active antimicrobial compounds. The findings of this study are therefore in agreement with the previous reports by Cowan (1999). Other authors reported the potential of aqueous extracts of *Hibiscus sabdariffa*, *Punica granatum*, and *Eucalyptus globulus* to protect potato plants against bacterial wilt caused by *R. solanacearum* under greenhouse and field conditions (Hassan *et al.*, 2009).

Darout *et al.* (2000) reported the antimicrobial action of the aqueous extracts to components such as thiocyanate, nitrate, chlorides and sulfates besides other water soluble substances present in the plant material. Therefore organic solvents were used for extraction of potent antibacterial compounds. Therefore the results are in conformity of previous studies which have reported that organic solvents like methanol are better solvents for more consistent extraction of antimicrobial substances from medicinal plants compared to water (Karaman *et al.*, 2003; Emad *et al.*, 2009; Parekh *et al.*, 2005).

Bioactive compounds may interfere with bacterial cell wall synthesis (Rasooli and Mirmostafa, 2002), protein synthesis, and nucleic acid synthesis, breaking the peptide bonds, acting as chelating agents, interfering with metabolic functions (Arekemase *et al.*, 2011) and preventing the utilization of available nutrients by the microorganisms thus hampering its survival (Gobalakrishnan *et al*., 2014).

Activity index (AI) of the plant extracts in various solvents was determined and AI of 50% and more was considered as active product for further study. Ethanol extracts of *P. guajava* (79%), *G. indica* (63.3%), *A. auriculiformis* (62.5%), *T. indica* (57.5%), *C.*

zevlancium (54.1%), *M. pudica (*51.6%), *A. occidentalis (*50.8%) and *C. gigantea* (50%) showed AI of 50% and more. Methanol extracts of *G. indica* (75%), *P. guajava* (70%), *A. occidentalis (*61.6%), *T. indica* (54.1%) and *L. intermis* (51.6%) were effective. Ethyl acetate extracts of *C. flexuosus*, *B. diffusa* and *C. zevlancium* showed AI of 79%, 75% and 61.6% respectively. Acetone extracts of *T. indica* (76.6%), *P. guajava* (70.8%), *A. auriculiformis* (59.1%) and *G. indica* (51.6%) showed AI of 50% and more. Dichloromethane and diethyl ether extracts showed activity index less than 30% (Table 5.2).

Table 5.2 Activity index of plant extracts inhibiting *R. solanacearum* Race 1 biovar 3 Rs 08-17

No.	Plant species	Activity Index (%)						
		80% Ethanol	Methanol	80% Ethyl Acetate	Acetone	DCM	DEE	Chloroform
1	*M. koenigii (leaf)*	26.6	0.0	0.0	0.0	0.0	0.0	25.8
	M. koenigii (Root)	22.5	0.0	0.0	0.0	0.0	15.0	3.3
2	*E. odorata*	20.8	25.0	0.0	0.0	0.0	0.0	0.0
3	*T.occidentalis*	20.0	31.6	37.5	28.3	0.0	0.0	0.0
4	*A. indica*	31.5	0.0	22.5	0.0	0.0	0.0	8.3
5	*O. tenuiflorum*	37.5	27.5	32.5	0.0	0.0	0.0	22.5
6	*A. vasica*	36.6	0.0	0.0	15.0	17.5	0.0	10.0
7	*G. indica*	63.3	75.0	23.3	51.6	0.0	24.1	7.5
8	*M. oleifera*	37.5	25.8	35.0	20.0	20.8	30.0	9.1
9	*S. asoca*	31.5	0.0	24.1	23.3	20.0	0.0	5.0
10	*Hibiscus sp.*	30.0	19.1	0.0	0.0	19.1	15.0	0.0
11	*J. curcas*	23.1	19.3	0.0	0.0	0.0	0.0	11.2
12	*A. marmelos*	31.6	26.6	9.1	31.6	28.3	0.0	7.5
13	*C. papaya*	30.0	35.0	7.5	25.8	0.0	0.0	15.8
14	*A. occidentalis*	50.8	61.6	24.1	0.0	0.0	26.2	10.0
15	*O. basilicum*	30.0	20.0	27.5	21.6	22.5	0.0	32.5
16	*C. zevlancium*	54.1	30.0	61.6	30.0	28.3	22.5	28.3
17	*M. fragrans*	22.5	0.0	0.0	0.0	18.3	0.0	0.0
18	*Glyricidia sp.*	30.0	24.1	31.6	0.0	0.0	0.0	27.5
19	*Z. officinale*	31.6	30.0	30.8	0.0	0.0	0.0	24.1
20	*P. guajava*	79.0	70.0	30.0	70.8	0.0	0.0	35.0
21	*L. intermis*	35.0	51.6	43.3	44.1	27.5	35.0	35.0
22	*A. carambola*	37.5	29.1	0.0	22.5	0.0	0.0	0.0

No.	Plant species	Activity Index (%)						
		80% Ethanol	Methanol	80% Ethyl Acetate	Acetone	DCM	DEE	Chloroform
23	*Alstonia sp.*	35.0	28.3	27.5	0.0	20.8	0.0	22.5
24	*C. gladiata*	35.8	25.0	29.1	40.0	0.0	0.0	0.0
25	*A. auriculiformis*	62.5	22.5	28.3	59.1	23.3	0.0	25.0
26	*Ricinus sp.*	27.5	31.6	25.0	0.0	0.0	0.0	61.6
27	*Physalis weed*	20.8	44.1	24.1	22.5	0.0	0.0	28.3
28	*M. pudica*	51.6	34.1	35.8	12.5	22.5	0.0	7.5
29	*C. gigantea*	50.0	37.5	38.3	35.0	5.0	0.0	5.0
30	*T. indica*	57.5	54.1	34.1	76.6	25.0	0.0	10.0
31	*B. diffusa*	37.5	41.5	75.0	30.0	22.2	0.0	0.0
32	*C. flexuosus*	32.5	0.0	79.0	29.0	25.0	0.0	0.0

AI (%) =100 x diameters of inhibition zone of extract ÷ diameters of inhibition zone of the standard antibiotic; DCM- Dichloro methane; DEE- Diethyl ether.

From these observations, it could be inferred that *G. indica*, *T.indica*, *P. guajava*, *A. occidentalis*, *M. pudica*, *C. zevlancium* and *A. auriculiformis* plants had lot of potential for further analysis. Therefore, plant extracts of these plant species were re-tested for their activity after one year of storage and it was found that the inhibitory activity of *G. indica* and *T. indica* remained highly consistent and unchanged (Table. 5.3).

Table 5.3 Antibacterial activity of plant extracts in different organic solvents against *R. solanacearum* Race 1 biovar 3 Rs-08-17

No.	Plant species	Inhibition zone of plant extracts against Rs-08-17 growth (mm)*							
		Ethanol		Methanol		Ethyl acetate		Acetone	
		1st Screening#	2nd screening¥	1st Screening#	2nd screening¥	1st Screening#	2nd screening¥	1st Screening#	2nd screening¥
1	*A. occidentalis*	20.3±0.27	15±0.0	24.6±0.27	7.0±0.0	9.6±0.27	10.0±0.0	0.0±0.0	0.0±0.0
2	*M. pudica*	20.6±0.0	9.0±0.0	13.6±0.54	7.3±0.57	14.3±0.0	9.0±0.0	6.0±0.0	8.6±1.15
3	*T. indica*	23.0±0.27	22.0±0.0	21.6±0.27	16.6±0.57	13.6±0.54	8±0.0	30.6±0.27	6.0±0.0
4	*A. auriculiformis*	25.0±0.0	0.0±0.0	9.0±0.0	0.0±0.0	11.3±0.0	9.3±0.57	23.6±0.0	21.0±0.0
5	*P. guajava*	31.6±0.27	15.3±0.57	28.0±0.47	19.66±0.57	12.0±0.0	13±0.0	28.3±0.54	0.0±0.0
6	*C. zevlancium*	21.6±0.27	8.3±0.57	12.0±0.0	6.0±0.0	24.6±0.27	12.3±0.57	12.0±0.0	0.0±0.0
7	*G. indica*	25.3±0.27	22.3±0.57	30.0±0.0	23.6±0.57	9.3±0.27	8.3±0.57	20.6±0.54	18±0.0

*The data represents the mean values of three replicates with (±) standard deviation; Streptomycin (50 µg μL^{-1}) control recorded 40 mm inhibition zone; Zone of inhibition of plant extract was measured after subtracting zone of inhibition if any by the corresponding solvent; #1st screening of the plant extracts; ¥2nd screening of plant extracts after a period of 1 year.

After the careful consideration on the efficiency of bacterial inhibition and the availability of plant species, *G. indica* and *T. indica* were selected for further characterization. Both the plant species grow abundantly in western India and their usefulness to mankind is known for centuries. Varalakshmi *et al.* (2010) reported the antimicrobial properties of *G. indica* on certain microbes. *T. indica* has broad spectrum antibacterial activity (Nwodo, 2011) due to the presence of polyphenols in their leaves (Arya, 1999). However, their antimicrobial activity against *R. solanacearum* was not known.

5.3.3. Phytochemical analysis

The phytochemical compounds present in *G. indica* and *T. indica* plant species which are responsible for antibacterial activity was not studied. Hence it was decided to explore the antibacterial compounds in these plant species. Therefore, these two were selected for qualitative phytochemical screening by conventional standard protocols. The antibacterial activity of plant extracts due to the presence of various secondary metabolites such as alkaloids, flavanoids, steroids, terpenoids, tannins, phenols have earlier been reported against various phytopathogens (Surender *et al.*, 2009; Jayalakshmi *et aI.*, 2011; Govindappa *et al.*, 2011; Sunder *et at.*, 2011) and are known to possess antimicrobial activities (Ogunkunle and Tonia, 2006).

Phytochemical analysis revealed the presence of flavonoids in *G. indica* and tannins in *T. indica* (Table 5.4). Saponins, terpenoids and alkaloids were present in both the plant extracts (Fig 5.2). These phytochemical components with phenolic structures are known to be highly active against the pathogen and serves as plant defense mechanisms against pathogenic microorganisms (Das *et al.*, 2010).

Table 5.4 Phytochemical analysis of ethanol extracts of *G. indica*, *T. indica* and antibacterial activity of solvent-solvent partitioning fractions (F-I, F-II, F-III) against *R. solanacearum*

Characters	*G. indica*	*T. indica*
Phyto-chemicals*		
Flavonoids	+	-
Tannins	-	+
Saponins	+	+
Terpenoids	+	+
Alkaloids	+	+
Antibacterial activity of fractions#		
Chloroform (F-I)	2.0	4
n-butanol (F-II)	0	0
Ethanol (F-III)	21.5±0.70	20.5±0.70

*The experiment was repeated two times with two replications; (+) indicates presence (-) indicates absence of the chemical; # Zone of *R. solanacearum* inhibition (diameter in mm) by the ethanol fractions obtained by solvent-solvent partitioning; mean of three replications with (±) standard deviation.

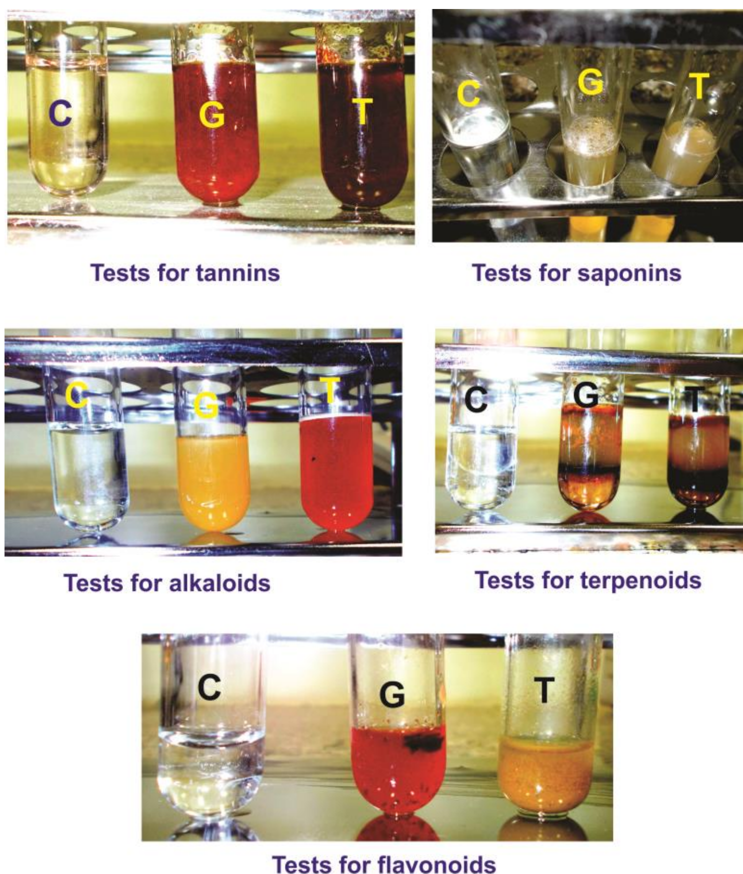


Fig. 5.2 Preliminary Phytochemical screening of Plant extracts of *G. indica* and *T. indica* for determining tannins, saponins, alkaloids, terpenoids, flavonoids. C-Control; G- *G. indica* extract; T-*T. indica* extract

Saponins are widely distributed amongst plants and it is believed to protect the plant against pathogens due to its antimicrobial activity (Anyasor *et al*., 2010). Tannins are known for their astringent property and antimicrobial activity (Cowan, 1999). Zhao *et al.* (2011) reported the antibacterial activity of flavonoids derived from *D. odorifera* against *R. solanacearum*.

The ethanol extracts were further subjected to solvent fractionation and all the three fractions were tested for *R. solanacearum* inhibition. Ethanol fraction (F-III) of *G. indica* and *T. indica* showed inhibition of 21.5 and 20.5 mm respectively (Table 5.4; Fig 5.3).

Lesser degree of inhibition (*G. indica*: 2 mm and *T. indica*: 4 mm) was showed by chloroform fraction (F-I). N-butanol fractions (F-II) did not inhibit the pathogen (Table 5.4). Therefore, it was decided to identify the compounds present in the ethanol fractions (F-III) of *G. indica* and *T. indica* by GC-MS analysis.

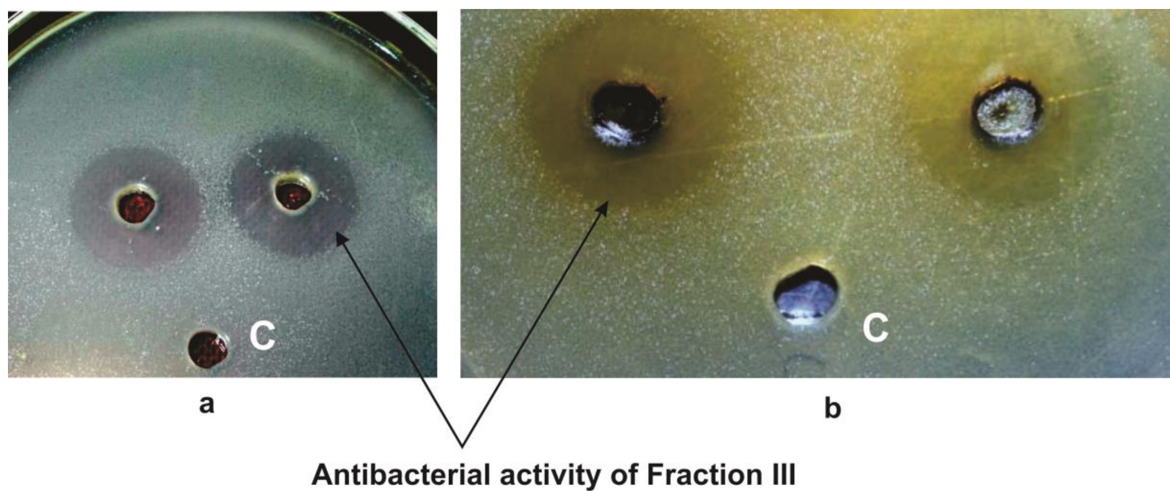


Fig. 5.3 Antibacterial activity of fraction III portion of (a) *G. indica* and (b) *T. indica* against *R. solanacearum* Rs-08-17 isolate displaying inhibitory halos, C-Control.

5.3.4. GC-MS analysis

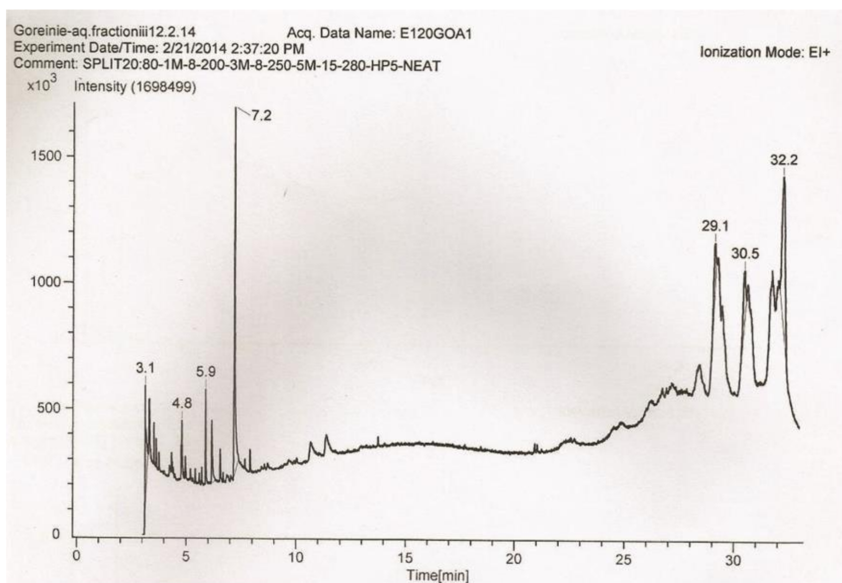
The active principles of fraction III of *G. indica* and *T. indica* are presented in Table 5.5 and 5.6 respectively, with their Retention time (RT), molecular formula (MF), molecular weight (MW) and peak area (%).

Fraction III extract of *G. indica* produced 9 peaks in GC-MS analysis as illustrated in Fig. 5.4 a. Out of these peaks; compounds were identified for 4 peaks corresponding to their respective retention time of 5.9, 7.2, 30.5 and 32.2. Two antimicrobial compounds *viz*. 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl, a flavonoid (Fig. 5.4 b) and 2-Furancarboxaldehyde,5 (hydroxymethyl), an aldehyde compound (Fig. 5.4 c) were identified based on mass spectrum.

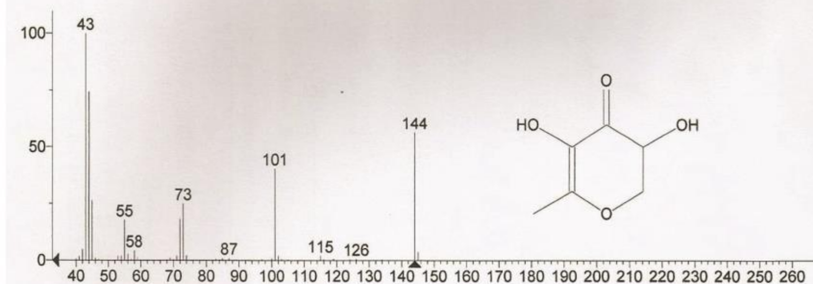
Table 5.5 Phytochemicals identified in the ethanol extracts of the leaves of *G. indica* by GC-MS

Peak no.	RT (min)	Peak width	Area (%)	Compound name	MF	MW	Compound nature	Activity***
1	3.1	0.124	8.3	-	-	-	-	-
2	3.3	0.045	3.8	-	-	-	-	-
3	4.8	0.027	3.5	-	-	-	-	-
4	5.9	0.026	3.7	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144.127	Flavonoid fraction	Antimicrobial, Anti-inflammatory, Anti proliferative
5	6.2	0.044	4.5	-	-	-	-	-
6	7.2	0.034	23.8	2-Furancarboxaldehyde, 5(hydroxymethyl)	$C_6H_60_3$	679.8	Aldehyde compound	Antimicrobial, preservative
7	29.1	0.10	7.9	-	-	-	-	-
8	30.5	0.12	10.1	Neo tigogenin	$C_{27}H_{44}O_3$	416.64	Steroid sapogenin	-
9	32.2	0.17	33.8	Spirostan-12-one, 3 hydroxy-(3β, 5α, 25R)	$C_{27}H_{42}O_4$	430.62	Steroidal sapogenin	Induction of apoptosis

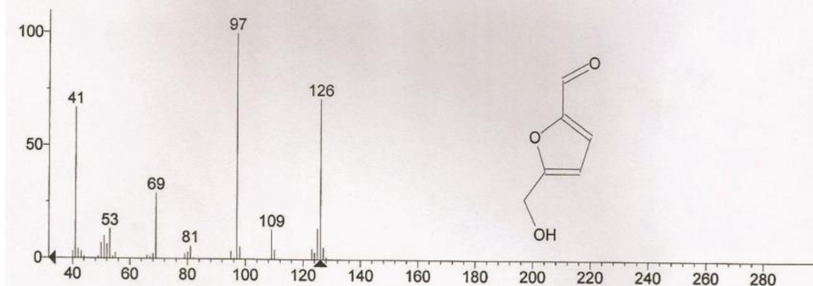
Retention time (RT), molecular formula (MF), molecular weight (MW) and peak area (%); ***Source: Dr. Duke's: Phytochemical and Ethnobotanical Databases (online databas



a



b



c

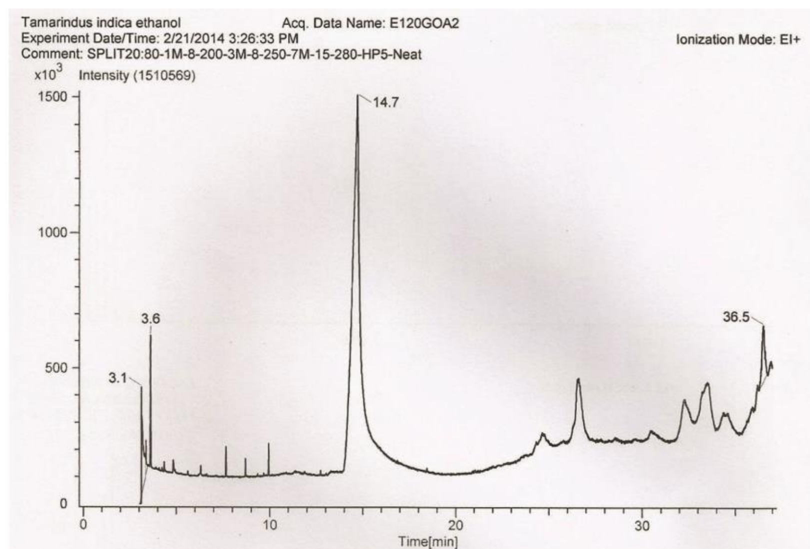
Fig. 5.4 (a) GC-MS Chromatogram showing chemical constituents of ethanol extract (F-III) of the leaves of *G. indica*; Mass spectrum of (b) 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (RT: 5.9) and (c) 2-Furancarboxaldehyde, 5(hydroxymethyl) (RT: 7.2)

Fraction III extract of *T. indica* produced 5 peaks in GC-MS analysis as illustrated in the Fig. 5.5 a. Out of these peaks; compounds were identified for 3 peaks corresponding to their respective retention time of 3.6, 14.7 and 36.5 (Table 5.6). The fraction revealed the presence of a carboxylic acid ester (Butanoic acid, butyl ester) and fatty acid ester compound (Myo-inositol, 4-C-methyl) based on mass spectrum (Fig. 5.5 b, c) with antimicrobial properties.

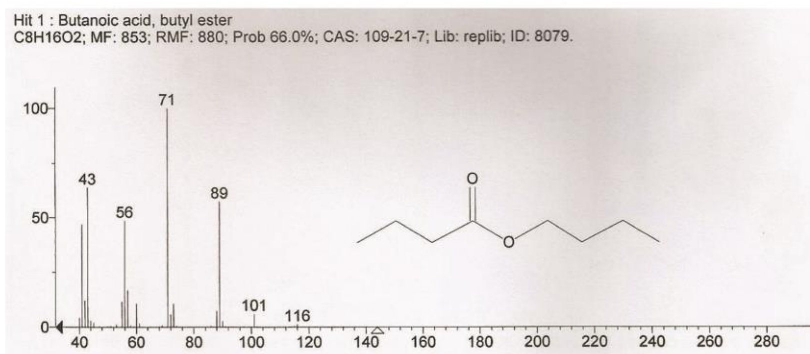
Table 5.6 Phytochemicals identified in the ethanol extracts of the leaves of *T. indica* by GC-MS

Peak no.	RT (min)	Peak width	Area (%)	Compound name	MF	MW	Compound nature	Activity***
1	3.1	0.082	27.6	-	-	-	-	-
2	3.4	0.033	4.1	-	-	-	-	-
3	3.6	0.025	10.4	Butanoic acid, butyl ester	$C_8H_{16}O_2$	144.21	Carboxylic acid ester	Antibacterial
4	14.7	0.099	26.9	Myo-inositol,4-C-methyl	$C_7H_{14}O_6$	194.18	Fatty acid ester	Antimicrobial, Antioxidant
5	36.5	0.18	30.8	Spirostan-12-one, 3 hydroxy-(3^{β}, 5α, 25R)	$C_{27}H_{42}O_4$	430.62	Steriod saponin	Induction of apoptosis

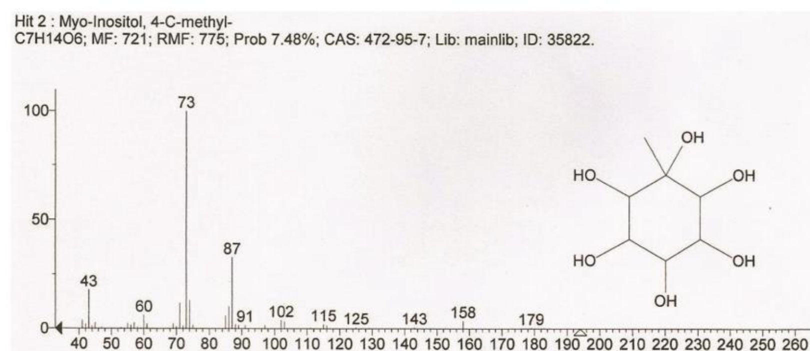
Retention time (RT), molecular formula (MF), molecular weight (MW) and peak area (%)
***Source: Dr. Duke's: Phytochemical and Ethnobotanical Databases (online database)



a



b



c

Fig. 5.5 (a) GC-MS Chromatogram showing chemical constituents of ethanolic extract (F-III) of the leaves of *T. indica*; Mass spectrum of (b) Butanoic acid, butyl ester (RT: 3.6) and (c) Myo-inositol,4-C-methyl (RT: 14.7)

This is the first report on the antibacterial effect of *G. indica* and *T. indica* against *R. solanacearum* and the presence of antimicrobial compounds in the leaf extracts.

Summary and Conclusion

1. *R. solanacearum* isolates were collected from Goa (n=33) and Maharashtra (n=8) from bacterial wilt infected eggplant (*Solanum melongena* L.), chilli (*Capsicum annum* L.) and tomato (*Lycopersicum esculentum* L.). *R. solanacearum* isolates produced typical irregular white fluidal colonies with pink centers on TZC medium. Identification of these isolates was further confirmed by PCR using Rs-759/760 primer set specific to the *R. solanacearum* species.
2. Biovar characterization indicated that all the *R. solanacearum* isolates belonged to biovar 3, except one isolate which was biovar 6. Further phylotype analysis indicated that the isolates belonged to Phylotype I.
3. Host range studies indicated that 98% of the isolates were pathogenic on eggplant, 88% isolates were pathogenic on tomato plants and 22% were pathogenic on chilli plants.
4. *R. solanacearum* isolates were genetically characterized using various molecular techniques viz. ARDRA, PCR-RFLP, rep-PCR fingerprinting and gene sequencing. ARDRA and PCR-RFLP techniques failed to show polymorphism amongst the phylotype I isolates. High degree of diversity among the *R. solanacearum* isolates was displayed when rep-PCR fingerprinting was used. Discrete classification of the *R. solanacearum* isolates based on host, geographical origin or the biovar was not observed.
5. Nucleotide sequence analysis of *egl* gene of 10 representative *R. solanacearum* isolates resulted in the assignment of sequevars to 3 of the isolates and the other 7 constitute unknown sequevars.

6. MLST analysis of 10 representative *R. solanacearum* isolates revealed the presence of six haplotypes, haplotype 1 being the dominant one comprising five isolates.
7. Virulence of *R. solanacearum* on eggplant seedlings indicated that the isolates caused BW in the range of 10-100%, exhibiting variations in their potential to cause the infection. Wilt incidence was faster in case of petiole inoculation as against the soil drenching method.
8. Motility assays revealed that all pathogenic wild type *R. solanacearum* isolates were motile and display twitching motility. Further these isolates exhibited a positive chemotactic response towards various sugars, amino acids and organic acids. However, the *eps* mutant strains did not possess twitching motility and were non-chemotactic.
9. All the *R. solanacearum* isolates including the *eps* mutant produced virulence factors viz. siderophores, cellulase and pectinase.
10. Characterization of EPS from the pathogenic wild type *R. solanacearum* isolate Rs-08-17 indicated that the EPS is acidic in nature. Further IR spectroscopy analysis revealed the presence of sugars, uronic acids and an aromatic functional group in the EPS produced by *R. solanacearum*.
11. Various environmental, soil and other factors influence the survival of *R. solanacearum.* Concentration of NaCl adversely affected the multiplication of *R. solanacearum in vitro* as well as in soil. Reduced EPS production was also noticed with increased concentration of salt. However, the increased salt

concentration had no effect on the disease incidence when eggplants were inoculated with *R. solanacearum*.

12. *R. solanacearum* failed to survive at temperatures below 20 °C and above 37 °C *in vitro* as well as in soil. BW incidence was recorded at 30°C to 37°C when eggplants were inoculated with *R. solanacearum.*
13. pH plays a role in the survival and multiplication of *R. solanacearum in vitro* and in soil. Better survival of *R. solanacearum* was recorded between pH 6.0 to 7.0 and the growth was suppressed at pH 5.0. BW incidence was high at pH 6.0 to 7.0 when eggplants were inoculated with *R. solanacearum.*
14. *R. solanacearum* was persistent in the soil with MHC of 25-50% but did not survive when above 75% MHC. BW incidence was high in soil with 0-50% MHC when eggplants were inoculated with *R. solanacearum.*
15. Population of *R. solanacearum* was high in the rhizosphere region than in the bulk soil at various temperature, pH and moisture conditions.
16. Thirty two plant species were screened to exploit their antimicrobial activity against *R. solanacearum* Rs-08-17 Race 1 biovar 3 isolate. Alcohol extracts of plant species showed better activity against *R. solanacearum* as compared to other solvents used. Activity index was higher in *G. indica* and *T. indica* as they were highly inhibitory to the pathogen.
17. Phytochemical analysis of crude extracts of *G. indica* and *T. indica* detected the presence of saponins, terpenoids, alkaloids, flavonoids and tannins.

18. GC-MS analysis of the inhibitory fractions of crude ethanol extract indicated the presence of antimicrobial components of 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl and 2-Furancarboxaldehyde 5 (hydroxymethyl) in *G. indica* and butanoic acid and Myo-inositol, 4-C-methyl in *T. indica*.

In Goa, a variety of solanaceous vegetables are cultivated throughout the year. Eggplant cultivation in Goa is severely affected due to bacterial wilt caused by *R. solanacearum*. *Agassaim* is the popular local cultivar found in Goa, highly susceptible to wilt and disease incidence. In this study it was observed that *R. solanacearum* isolates from Goa although phenotypically similar, are diverse in its genetic characteristics. This could be the major reason for the difference in virulence among the isolates as the bacterium is evolving gradually and continuously. For the successful infection of the host, *R. solanacearum* possess multiple virulence factors. The results from this study prove that EPS is one of the most important virulent factors responsible for the BW. Individual virulent factors alone may not be essentially necessary for causing the disease and therefore it remains unclear to what extent these pathogenic determinants contribute to the virulence of *R. solanacearum*. Environmental and soil factors have a very important role in determining the persistence of *R. solanacearum* in soil. Soils with poor water drainage, high salt concentration and extreme pH and temperature ranges are not favourable for survival of *R. solanacearum*. An extreme adaptation of *R. solanacearum* towards wide range of hosts, environmental factors and its survival mechanisms makes its management very difficult and hence a number of attempts have failed to manage the disease. Therefore, an eco-friendly approach was devised in this study by evaluating the crude plant extracts of *G. indica* and *T. indica* which showed the presence of promising

antibacterial product for controlling the bacterial wilt *in vitro*. These extracts need to be tried and tested in field experiments for analyzing their potential. This has opened up a new perspective in the management of BW caused by *R. solanacearum*. Further research is necessary to purify and test the antibacterial compounds against *R. solanacearum* so as to use them in the formulation of antibacterial agents for the BW control.

Appendix A

A. Media composition

A1. SMSA Selective medium (Engelbrecht, 1994 as modified by Elphinstone *et al*., 1996)

Ingredients	**g L^{-1}**
Casamino acids	1.0 g
Bacto Peptone	10.0 g
Glycerol	5.0 mL
Agar	15.0 g
Distilled water	1000 mL

Prepare 1/2 litre volumes of medium in one litre flask. Dissolve the ingredients and check the pH. Adjust pH, if necessary to 6.5 before autoclaving. *R. solanacearum* will not grow well on the medium at pH > 7.0. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C. Add 250 mL of melted media at a temperature of 50 °C.

Antibiotics	Final Conc. (ppm)	Volume
1% Polymyxin B sulfate	100.0 ppm	2.5 mL
1% Crystal violet	5.0 ppm	125 µL
1% tetrazolium salts	50.0 ppm	1.25 mL
1% Bacitracin	25.0 ppm	625 µL
0.1% Penicillin	0.5 ppm	125 µL
1% Chloramphenicol	5.0 ppm	125 µL
1% cycloheximide	100 ppm	2.5 mL

Dissolve the ingredients in 70 % ethanol to the given concentrations for the volume of medium prepared. Some ingredients, viz. polymixin B and chloramphenicol require slight warming and shaking.

Appendix B

B. Composition of stains and reagents

B.1. Stains

i) Gram stain reagents

Crystal violet

Solution A- 1g of crystal violet in 10 mL ethanol

Solution B- 0.8g ammonium oxalate dissolved in 80 mL distilled water

Mix Solution A and B and filter through Whatman filter paper No. 1.

Gram's Iodine

Dissolve 1g iodine and 2g Potassium iodide in 300 mL distilled water. Filtered through Whatman filter paper No. 1 (diameter =12.5 cm).

Safranine

2.5g Safranine was dissolved in 10 mL ethanol and made the volume up to 100 mL with distilled water and filtered through Whatman filter No.1.

Procedure for gram staining

Prepare the bacterial smear on a clean grease free glass slide and heat fix it. Flood the smear with crystal violet for 1.5 min and gently wash with running tap water, then flood the smear with Gram's iodine for a min. Gently wash with tap water and decolorize with 90% ethanol prepared in distilled water till colour oozes out. Counter stain using saffranine for 1 min. Wash with tap water, air dry the slide and then put a drop of oil on smear to examine under oil immersion lens of microscope.

B.2. Reagents

(i) Solution A (Sulphanilic acid)

Ingredients	**g L^{-1}**
Sulphanilic acid	8 g
Acetic acid (5 N)	One part of acetic acid added to 2.5 parts of distilled water
	Distilled water make final volume 1 L

(ii) Solution B (α -naphthylamine)

Ingredients	**g L^{-1}**
α-naphthylamine	5 g
Acetic acid (5N)	1 L

(iii) Mayer's reagent

1.358 g of $HgCl_2$ was dissolved in 60 mL of water. A solution of 5g of KI in 10 mL of H_2O was added to it and the final volume was made up to 100 mL.

(iv) 1 M NaCl

Dissolve 40 g of NaCl in 1000 mL of distilled water

Appendix C

C.1 Reagents for Genomic DNA isolation

1 M Tris HCl: 12.11g of Tris HCl in 100 mL of distilled water.

0.5 M EDTA: 14.6125g of EDTA in 100 mL of distilled water.

1 M Na_2HPO_4: 17.8g of Na_2HPO_4 in 100 mL of distilled water.

Proteinase K (10 mg mL^{-1}): 10 mg of Proteinase K in 1 mL of distilled water.

Lysozyme (20 mg mL^{-1}): 10 mg of lysozyme in 1 mL of distilled water.

20% SDS (Sodium Dodecyl Sulphate): 2g of SDS in 10mL of distilled water.

Chloroform: isoamyl alcohol (24:1): 24 mL of chloroform + 1mL of isoamyl alcohol.

70% ethyl alcohol: 70 mL of absolute ethanol + 30 mL of distilled water.

C.2 Genomic DNA extraction buffer

Components	Concentration
Tris HCl	100mM
EDTA	100mM
Na_2HPO_4	100mM
NaCl	1.5M
CTAB	1%
Proteinase K	20µg
Lysozyme	100µg
Distilled water	Make to 100ml

C.3 Gel loading dye (6X)

Component	Concentration
Bromo phenol blue	0.25%
Glycerol/sucrose	30%
Distilled water	100 mL

Appropriately diluted to 1X when added to DNA sample

C.4 Agarose gel electrophoresis

Component	Agarose concentration	
	0.8%	**1.5%**
Agarose	0.48 g	0.9g
1X TAE buffer	60 mL	60 mL
EtBr (10mg mL^{-1})	3 μL	3 μL

C. 5 Ethidium bromide solution (10 mg mL^{-1})

Component	Quantity
Ethidium bromide	10 mg
Distilled water	1 mL

Stock solution was prepared and kept cool and dark places. The final concentration used for agarose gel was 0.5 μg mL^{-1}

Appendix D

D. Buffers

D.1. Composition of acetate buffer (pH 6.0)

(i) Preparation of 0.1 M acetic acid**:** Dilute 6.0g of glacial acetic acid to 1000 mL with distilled water

(ii) Preparation of 0.1 M sodium acetate tri-hydrate: Dissolve 13.6g in 1000 mL distilled water

To prepare 1000 mL 0.1 M Acetate buffer: Mix 52.2 mL of 0.1M acetic acid and 947.8 mL of 0.1 M sodium acetate

D.2. Composition of phosphate buffer (pH 5.3)

(i) Preparation of 0.2 M $Na_2HPO_4.2H_2O$: Dissolve 35.61 g of $Na_2HPO_4.2H_2O$ in 1000 mL of distilled water

(ii) Preparation of 0.2 M $NaH_2PO_4.H_2O$: Dissolve 27.6 g of $NaH_2PO_4.2H_2O$ in 1000 mL of distilled water

To prepare 1000 mL 0.2 M Phosphate buffer: Mix 770 mL of $Na_2HPO_4.2H_2O$ and slowly add $NaH_2PO_4.H_2O$ and adjust the pH to 5.3 using pH meter.

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Research Publications

- **Sapna Gaitonde** and R. Ramesh. 2014. Genetic characterization of *Ralstonia solanacearum* infecting eggplant *Solanum melongena* L. from Goa and Western region of India. *International Journal of Current Science* **12**: E 128-139
- Ramesh R, **Gaitonde S**, Achari G, Asolkar T, Singh NP, Carrere S, Genin S, 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 Announcement* **2**(3):e00323-14. doi:10.1128/genomeA.00323-14.
- Raman Ramesh, Gauri A. Achari and **Sapna Gaitonde**. 2014. 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. DOI 10.1007/s10658-014-0487-5
- R. Ramesh, J. Anthony, T.C.D. Jaxon, **S. Gaitonde** and G. Achari. 2011. PCR-based sensitive detection of *R. solanacearum* from soil, eggplant, seeds and weeds, *Archives of Phytopathology and Plant Protection* **44**:19, 1908-1919.
- Barbuddhe, S.B., Ramesh, R., Doijad, S.P., Poharkar, K.V., Achari, G., **Gaitonde, S.** and D'Costa, D. 2011. Training Manual on "Molecular subtyping of microbes using pulsed field gel electrophoresis. ICAR Research Complex for Goa. P40
- Sarita Nazareth, **Sapna Gaitonde** and Tabitha Marbaniang. 2012. Metal resistance of halotolerant fungi from mangroves and salterns of Goa, India. *Kavaka* **40**: 15-21

Oral and Poster Presentations

- Oral presentation entitled ‘Pathogenic characterization of *R. solanacearum* strains from Goa’ at Symposium on “Recent trends in disease management of Horticultural crops”, Indian Phytopathological Society (West zone), Dr. B.S.K.K.V., Dapoli, held during 19-20th October, 2011
- Oral presentation entitled ‘Genetic characterization of *R. solanacearum* strains from Goa’ at Symposium on “Microorganisms and Plant health”, Indian Phytopathological Soceity (North-East zone), Guwahati University, Assam, held during 3-5th November, 2011
- Poster presentation at Seminar on “Role of Microbes in Health, Agriculture and Industry” at Alagappa University, Karaikudi, TN, sponsored by UGC and DST held during 26-27 March, 2012
- Poster presentation entitled “*R. solanacearum* biovar 3 isolates differ in their virulence on eggplant. National symposium on “Microbial diversity and its applications in health, agriculture and industry”. 4-5th March, 2011 held at ICAR Research Complex for Goa, Old Goa, Goa, India pp 50-51 (Extended summary).
- International Seminar on “Strategies for improving livelihood security of Rural poor” held during 24-27th September, 2008

Abstracts

- **Gaitonde, S**. and Ramesh, R. 2011. *R. solanacearum* biovar 3 isolates differ in their virulence on eggplant. National symposium on "Microbial diversity and its applications in health, agriculture and industry". 4-5th March, 2011 held at ICAR Research Complex for Goa, Old Goa, Goa, India pp 50-51 (Extended summary).
- Ramesh, R., **Gaitonde, S.** and Achari, G. 2011. Genetic diversity of *R. solanacearum* from Goa. National symposium on "Microbial diversity and its applications in health, agriculture and industry". 4-5th March, 2011 held at ICAR Research Complex for Goa, Old Goa, Goa, India pp 33-34 (Extended summary).
- Ramesh, R., Achari, G, **Gaitonde, S** and Singh, N. P. 2011. Detection of *Ralstonia solanacearum* from soil by BIO-PCR. 5th International Bacterial Wilt Symposium (Abs), China.
- G. A. Achari, R. Ramesh and **S. Gaitonde**. 2012. Diversity, Antagonistic and Growth Promotion Ability of Xylem Residing Bacteria Isolated from Eggplant and Chilli. Symbiosis and Microbial Interactions symposium held at USA.

Training programs

- National workshop on “Molecular subtyping of microbes using PFGE” sponsored by DBT, GOI held during 20-23 August, 2011 at ICAR, Goa
- Training programme on “Entrepreneurship and Skill Development Programme on Biotechnology (Bio-fertilizers and Bio-pesticides)” sponsored by MS-ME Development Institute, GOI, Goa from 26th March to 22nd April, 2008.
- Awarded a Certificate as the “Most determined delegate” at IMTR Training Programme “Managing Change in Government Organisations (Sponsered by DST, GOI) held during 27th Feb to 3rd March, 2012 at International Centre, Donapaula, Goa