Isolation and characterization of *Ralstonia solanacearum* (Smith) Yabuuchi et al. causing bacterial wilt of tomato from sub-Himalayan West Bengal and its management

Thesis submitted to the University of North Bengal
for the award of Doctor of philosophy in
Botany

Submitted by
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September, 2021

Declaration

I, Hrisikesh Mandal hereby declare that the work embodied in my thesis entitled "**ISOLATION AND CHARACTERIZATION OF RALSTONIA SOLANACEARUM (SMITH) YABUUCHI ET AL. CAUSING BACTERIAL WILT OF TOMATO FROM SUB-HIMALAYAN WEST BENGAL AND ITS MANAGEMENT**" *has been carried out by me under the supervision of* ***Prof. Aniruddha Saha****, Department of Botany, University of North Bengal and under the co-supervision of* ***Prof. Dipanwita Saha****, Department of Biotechnology, University of North Bengal for the award of the Degree of Doctor of Philosophy in Botany. I also declare that, this thesis or any part thereof has not been submitted for any other degree/Diploma either to this or other University.*

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Abstract

Tomato is one the popular vegetable crop grown throughout world. Northern plains of West Bengal (popularly known as sub-Himalayan West Bengal) produce large quantity of tomato. Among the seven districts of North Bengal three districts are famous for tomato production. Those three districts are Coochbehar, Uttar-Dinajpur and Dakshin-Dinajpur. Several diseases of the crop have been reported from this part. Although most of the diseases are caused by fungi, but substantial losses of crops have been reported by bacterial wilt caused by *Ralstonia solanacearum*. The farmers of the present study area depend mostly on synthetic pesticides for the disease management. Synthetic pesticides alter the natural soil-microflora and also pollute the environment. The residual chemicals of the crop also create health hazard to us. Although a large number of research works have been done on 'Tomato-Ralstonia' interaction throughout the world but very less number of works is available in literature from the present study area.

Considering the above the present work was planned to isolate and identify the bacterial wilt pathogens from the present study area and management of the disease by eco-friendly ways. The objectives of the study are as follows. 1) Isolation of *Ralstonia solanacearum* from soils of infected plants of North Bengal. 2) Pathogenesity test of selected isolates and assessment of disease. 3) Morphological studies of selected bacteria and electron microscopy. 4) Biochemical characterization and identification of the isolates. 5) Molecular identification of selected isolates following 16S rRNA. 6) PCR amplification and sequencing of *fliC* gene of some selected isolates. 7) Isolation of antagonistic bacteria and their identification. 8) Control of the disease by biocontrol agents and botanicals. 9) Induction of defense related enzymes by some abiotic inducers.

At first, 50 different locations of three districts of sub-Himalayan West Bengal (Coochbehar, Uttar Dinajpur and Dakshin Dinajpur) were surveyed for

occurrence of the disease in tomato. On the basis of preliminary survey 26 different locations were found to be prone to bacterial wilt disease. Ten locations of Haldibari and three locations of Ghoksadanga both of Coochbehar district were found to show severe bacterial wilt disease symptoms in the cultivated tomatoes. Eight locations of Balurghat of Dakshin-Dinajpur district and four locations of Durgapur of Uttar-Dinajpur district were also found to show severe wilt symptoms during survey.

All the 26 isolated bacteria were assigned isolate codes and were subjected to confirmation of Koch's postulations based on pathogenicity tests. From the pathogenicity test results 3 bacteria were found to be highly pathogenic to moderately high pathogenic. Thirteen bacteria was pathogenic and 10 bacteria were weakly pathogenic. Three most virulent isolates were subjected to molecular studies and were reassigned codes as RSG01, RSG02 and RSG03 before submission of gene sequences to GenBank.

In pathogenicity test 'PKM-1' was most susceptible variety and 'Rashmi' was least susceptible. Varieties 'Vashali' and 'Rupali' were also susceptible but less susceptible than 'PKM-1' variety. Host range test was conducted to check infectivity of the most virulent isolate of tomato to three different solanaceous plants such as potato, Brinjal and Chilli. Potato showed mild susceptibility but Brinjal and Chilli plants did not show any disease symptoms.

Colony morphology of the 26 isolated *R. solanacearum* was mostly smooth, white and fluidal. All the isolated bacteria were non-spore producing, rod shaped and occurred in single or in pairs. In broth all the cultures were turbid with pellicle and sediments. Colony morphology of the three most virulent isolates was also studied on CPG medium. The shape of colonies of the three most virulent isolates (RSG01, RSG02 and RSG03) was irregular and round. Colour of the colonies became reddish to deep red but surface was smooth and milky. In motility medium all the isolated bacteria showed their motile nature.

From the scanning electronic microscopic (SEM) figures, the surface topography of the three virulent isolates (RSG01, RSG02 and RSG03) was more

or less smooth with some depressions. The size and shape of the three bacteria were also determined from the SEM figures. Shape of bacteria was elongated in size.

All the 26 isolated pathogens were Gram negative and identified as *Ralstonia* sp. Although all the bacteria were *Ralstonia* but there were minor differences in biochemical characteristics. From the carbohydrate utilization tests (conducted for the 3 most virulent isolates) it was found that RSG01 isolate was Methyl red, VP, Glucose, Adonitol and Lactose positive. RSG02 isolate was VP, Adonitol and Manitol positive. But RSG03 was VP, Glucose, Adonitol and Manitol positive. This also indicates presence of minor differences among the virulent isolates.

PCR amplification of 16S rDNA was done and the expected amplicons were detected on agarose gels. PCR products of three selected virulent bacterial isolates T6/RSG01, D3/RSG02 and D4/RSG03 were sent to Xcelris Genomics Ltd. for sequencing. Annotation and BLAST analysis were done and deduced amino acid sequences were submitted to GenBank and necessary accession numbers were procured.

The nucleotide sequence of RSG01 showed sequence similarity with some Indian isolates but sub-clustered separately. Identity of the bacteria was confirmed as it clustered with *Ralstonia solanacearum*. Sequence of RSG01 (accession no. KC237236) of the present study showed closest similarity with Indian isolate 'accession no. KP017457'.

The nucleotide sequence of RSG02 and RSG03 clustered together with clustered with Indian isolates. The closest Indian isolate was 'accession no. KM502217'. Indian isolates were also clustered with some USA, Thailand and Australian isolates.

A specific and sensitive PCR detection method of *Ralstonia solanacearum* using *fliC* gene was established by Schonfeld *et al.* (2003). In the present study nucleotide sequence identity and phylogenetic tree (based on *fliC* gene) was constructed and it was found that the RSG01 and RSG03 clustered together in

a sub group. That sub-group again clustered with RGS02. *fliC* gene sequence of RSG02 isolate was very much close to USA isolates accession nos. DQ657703 and DQ657701 of GenBank. *fliC* gene sequence of RSG01 and of RSG03 isolates were very much close to Indian isolate 'accession no. KF920693' and Japanese isolate 'accession no. KF275630' as recorded in GenBank. From the phylogenetic tree of *fliC* gene, RSG01 and RSG03 were very much close to each other (98-99 % similarity). 94% similarity was found when the cluster was compared with RSG02 isolate.

Three antagonistic bacteria (coded as HS01, HS02 and HS03) were isolated from soil. Among the three bacteria isolate HS01 showed best antagonism and restricted growth of *R. solanacearum* (isolate RSG01) significantly.

All three bacteria were Gram positive and rod shaped. From the biochemical tests it was also found that the bacteria were very much like *Bacillus* sp. Out of the three antagonistic bacteria, the most antagonistic bacteria were selected for molecular identification by PCR based method. After sequence identity and phyllogenetic tree construction the organism (isolate HS01) was identified as *Bacillus cereus.* The sequence of the 16S ribosomal RNA gene (partial) was submitted to GenBank and accession no. of the sequence (KC959841) was procured from GenBank.

After the characterization of the antagonistic bacteria they were considered to be tested for disease management in whole plants in pots and also in fields. *Bacillus cereus* isolate HS01 was best among the tested antagonists when applied separately. It could check wilt disease up to 20 days in pots. In field condition only isolate HS01 could check the disease significantly.

Trichoderma harzianum, a well known biocontrol agent was also procured and was tested against *R. solanacearum* both by *in vitro* (Dual culture technique) and *in vivo* (by application in whole plant). *T. harzianum* could check the growth of *R. solanacearum* completely when tested *in vitro*. *T. harzianum*

could reduce the disease (wilting index) up to 80% in sterilized soil in potted plants.

Eight plant extracts have been tested for their potentiality to check the growth of *R. solanacearum. Zingiber officinale, Azadirachta indica* and *Camellia sinensis* could inhibit the growth of the *R. solanacearum* significantly in agar cup bioassay (*in vitro* tests). Out of the three potential plant-extracts, *Camelia sinensis* leaf extract was selected for the *in vivo* studies. *Camellia sinensis* leaf extract could successfully control *R. solanacearum*-caused wilt in tomato plants grown in pots.

Three chemical inducers (BABA, SA, and ABA) were used for the purpose of defense induction in tomato against *R. solanacearum* (isolate RSG01). SA and BABA significantly induced resistance in tomato plants and also increased activity of the three defense related enzymes. ABA showed least activity in defense induction in tomato against *R. solanacearum*.

In conclusion, the present study reports several new findings. Isolation and identification of 26 pathogenic *Ralstonia solanacearum* isolates were done from tomato plants of North Bengal. Three different antagonistic bacteria (*Bacillus cereus*) have been isolated and identified. *Trichoderma harzianum* found to be potential in controlling the disease. One of the botanical (*Camellia sinensis* leaf extract) found to be effective in controlling the disease. Alternatively SA and BABA also could reduce the disease following induction of defense in tomato plants.

Preface

Origin of tomatoes as food dates back to around 700 A.D. by the Aztecs. It is told that tomato in wild and cultivated form came into knowledge from the mountains of Andes presently in the countries of Peru, Ecuador, and Bolivia. In India, it is believed, to be introduced by the Portuguese in 16th century but largely cultivated in British India during 18th century. In 2019–20 fiscal year India ranked second in terms of total tomato production (above 20 million tons).

Large quantity of tomatoes is destroyed annually due to pathogens like fungi, bacteria and viruses. Severe economical losses have been experienced by the farmers throughout world. In India, marginal farmers often face severe loss due to pathogen attacks in tomatoes. In the present study area bacterial wilt is very common and need to be studied scientifically.

Pesticide application to control bacterial diseases is very common practice to secure the worldwide food supply. But random, uses of pesticides pollute soil and also give rise to resistant and virulent bacterial strains. There is a need for new environment friendly pesticides and/or plant defense inducers to control the disease to save production loss of tomato.

Considering the above the present study were taken into consideration to find out the different strains of bacterial wilt-pathogens from study area. Their characterization and pathogenicity were also given priority to be determined. Few indigenous biocontrol bacterial strains were isolated and characterized. Some defense inducers have also been tested for their efficacy to induce resistance in susceptible tomato plants. Thus, the present study is directed towards pathogen isolation, characterization and management of the bacterial wilt disease in tomato.

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Abbreviations

µg	Microgram	**L**	Litre
µl	Microlitre	**LB**	Luria-Bertani
µm	Micrometre	**M**	Mole
^{o}C	Degree Celcius	**mAmp**	Milliampere
ABA	abscisic acid	**MEGA**	Molecular evolutionary genetics analysis
avr	avirulence	**mg**	Milligram
BABA	β-amino butyric acid	**min**	Minutes
BLAST	Basic local alignment search tool	**ml**	Millilitre
		mm	Milimetre
BLASTn	Nucleotide BLAST	**mM**	Milimole
bp	Base pair	**M-MuLV**	Moloney murine leukemia virus
cm	Centimetre	**MPKs**	mitogen-activated protein kinases
CTAB	Cetyl trimethyl ammonium bromide	**mRNA**	Messenger RNA
DNA	Deoxyribonucleic acid	**N**	Normal
dNTPs	Deoxyribonucleotide triphosphates	**NCBI**	National Centre for Biotechnology Information
EC	Enzyme class	**ng**	Nanogram
EDTA	Ethylenediamine tetra acetic acid	**nm**	Nanometer
g	Gravitational force	**No.**	Number
gm	Gram	**nt**	Nucleotide
h	Hour	**PAL**	Phenylalanine ammonia lyase
ISR	Induced systemic resistance	**PDA**	Potato dextrose agar
ITS	Internal transcribed spacer	**PDB**	Potato dextrose broth
kb	kilo bases	**PCR**	Polymerase chain reaction
PPO	Polyphenol oxidase	**SE**	Standard error
PVP	Polyvinyl pyrrolidone	**SEM**	Scanning Electron Microscopy
RNA	Ribonucleic acid	**TAE**	Tris acetate EDTA
rpm	Rotation per minute	**UV-VIS**	Ultraviolet-Visible
rRNA	Ribosomal RNA	**V**	Volt
SA	Salicylic acid	**v/v**	Volume by volume
SAR	Systemic aacquired resistance	**w/v**	Weight by volume
SDT	Sequence demarcation tool	**wt**	Weight

Content

Chapter-1

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most economically important vegetable crops and it is cultivated worldwide for its fresh fruits. Tomato is a short duration, high yielding crop and belongs to family Solanaceae. Cultivation of tomato has been increased over the years due to its popularity and economic importance (Elphinstone *et al.* 1996). Tomato is a rich source of minerals, vitamins, organic acids, essential amino acids and dietary fibres. Tomato also contains lycopene and beta carbonate pigments (http://www.indiagronet.com/tomato/resources). In India, the most promising states for cultivation of tomato are Bihar, Uttar Pradesh, Orissa, Karnataka, Punjab, West Bengal and Assam. Previously tomatoes were grown only once in growing season, but presently tomato are grown round the year.

Like many other crop plants tomato also suffer from several diseases. Most of the diseases caused by fungi but bacterial wilt of the plants also cause a substantial loss. Wilt is a disease where loss of turgor in a plant or plant part is happened (Windhan and Alan, 2003). Bacterial wilt can cause severe yield reduction, in the range 30-80%, however, it is geographically restricted (Clark and Moyer, 1988). The causal organism of bacterial wilt is *Ralstonia solanacearum. R. solanacearum* is supposed to be a soil born bacterium originating from the tropics, subtropics and warm temperate regions (Hayward, 1991). *R. solonacearum* is a serious pathogen causing bacterial wilt in solanaceous vegetables in India, such as tomato, potato, banana, eggplants and some ornamental plants (Hayward, 1994).

In case of wilt disease the vascular tissues in the lower stem of plants show a dark brown discoloration. This symptom is similar to that of some fungal diseases. A cross section of the stem of a plant with bacterial wilt produces white, milky strands of bacterial cells in clear water. This ooze distinguishes the wilt caused by bacterium from that caused by fungal pathogens (Leppal *et al.* 2004). On all infected plants, bacterial streaming (ooze) may occur upon placing cut main stem material in a test tube with water. If vascular tissue is collapsed, sunken skin lesion will also appear (Swanson *et al.*, 2005).

The pathogen *Ralstonia solanacearum* has been classified in five biovars according to carbon source utilization and six races based on host range (Hayward *et al.* 1990). There is no general correlation between races and biovars, however biovar 2 strains are almost always race 3 (and vice versa). The five races of *R. solanacearum* have different host ranges and geographic distributions. Race 1 is a poorly-defined group with a very wide host range and is endemic to the southern United States. Race 2 principally attacks bananas, and is found mainly in Central America and Southeast Asia. Race 3 is distributed worldwide and has primarily been associated with potato. Race 4 affects ginger in much of Asia and Hawaii, and race 5 affects mulberries in China. Recently a more phylogenetically meaningful system has classified *R. solanacearum* into four phylotypes roughly corresponding to geographic origin (Fegan and Prior, 2005).

Bacterial wilt of tomato caused by *R. solanacearum* is a major disease in winter season. A specific and sensitive PCR detection method that uses primers targeting the gene coding for the flagella subunit (*fliC*) was established. Based on the first *fliC* gene sequence of *R. solanacearum,* strain K60 is available at GenBank, the Ral_*fliC* PCR primer system was designed and this system yielded a single 724 bp product with the DNAs of all *R. solanacearum* strains tested (DeShar *et al.* 1997; Hales *et al.* 1998; Shah *et al.* 2000).

Bacterial wilt pathogenesis is incompletely understood, genetic and molecular studies have shown that contributing factors are controlled by environmentally responsive regulatory cascades and that disease development depends on the action of the Type II and Type III protein secretion system. Secreted proteins are central to the pathogenesis of pathogenic bacteria (Schell, 2000 and Boucher, 2004).

Many plant species produce volatile essential oil compounds. These oils are considered to play a role in host defence mechanisms against plant pathogens. Essential oils and their components, usually from medicinal plants, have been recognized as having antifungal effects, but their efficacy

as a biofumigant on *R. solanacearum* has not been studied widely. However, some greenhouse experiments were conducted to determine the effectiveness of plant essential oils as soil fumigants to manage bacterial wilt in tomato (Kucharek, 1998; Mihaliak *et al.* 1991 and Wisniewski, 1997). Preliminary *in vitro* and greenhouse experiments conducted with several plant essential oils and their components showed the significant efficacy against *R. solanacearum* and against several soilborn fungi of tomato (Rich, 1999 and Mitchell, 2000).

With the above informations it was considered worthwhile to study the interactions of the *Ralstonia solanacearum* and *Lycopersicon esculentum* in sub-Himalayan north Bengal, where large quantity of tomato is produced. It was observed, during the present study, that bacterial wilt was a major constraint for successful cultivation of tomato in north Bengal (Fig 1.1). In addition it was also considered to suggest some control measures against the pathogen by using botanicals and biocontrol agents if any. Hence, the following objectives have been taken into consideration for the present study.

With the above information it was considered worthwhile to study the interactions of the *Ralstonia solanacearum* and *Lycopersicon esculentum* in sub-Himalayan West Bengal, where large quantity of tomato is produced. It was also considered to suggest some control measures against the pathogen by using botanicals and biocontrol agents if any. In addition, management of bacterial wilt disease (caused by *Ralstonia solanacearum*) by some abiotic inducers have also been taken into consideration. Hence, the main objectives of this study are as follows.

Objectives:

- Isolation of *Ralstonia solanacearum* from soils of infected plants of North Bengal.
- Pathogenesity test of selected isolates and assessment of disease.
- Morphological studies of selected Bacteria and Electron microscopy.
- Biochemical characterization and identification of the isolates.

- Molecular identification of selected isolates following 16S rRNA.
- PCR amplification and sequencing of *fliC* gene of some selected isolates.
- Isolation of antagonistic bacteria and their identification.
- Control of the disease by biocontrol agents and botanicals.
- Induction of defense related enzymes by some abiotic inducers.

Fig. 1.1: a) Infected young plants in farmers field b) Infected plants in farmers field.

Chapter-2

LITERATURE REVIEW

Major factor for limiting the production and market value of horticultural crops is their susceptibility to diseases. Tomato plants also face similar limitations of yield and commercial value due to diseases caused by several pathogens. To control the diseases of tomato, it is necessary to understand the different aspects of pathogens and hosts along with their interactions. Recent advanced initiatives in plant pathology have paved the way for development of new innovative techniques to control crop diseases. Molecular events of pathogenesis and induced systemic resistance have been recognized as the present era of plant pathology (Vidyasekharan, 1988). In addition to induced systemic resistance, biological control and control of diseases by botanicals have also gained much importance due to recent worldwide awareness on negative impact of chemical fungicides. Therefore, present research works of disease control of plants have also been focused towards eco-friendly mode of control that is benign to environment.

At the onset of the present research work, it was considered worthwhile to review the research findings of the previous researchers in a selective manner rather than in a comprehensive way. The observations of recent past have been presented briefly in the following paragraphs. For convenience, the observations have been grouped into some aspects. The different aspects of this literature review are as follows:

2.1: Pathogen (*Ralstonia solanacearum*)

2.1.1: Taxonomy.

2.1.2: Dissemination.

2.1.3: Detection and Identification.

2.1.4: Conditions favourable for development.

2.1.5: Distribution and host range.

2.2: Disease

2.2.1: Bacterial Wilt.

2.2.2: *Fusarium* Wilt.

2.3: Loss

2.4: Other diseases caused by *Ralstonia solanacearum*

2.5: Virulence

2.6: Serological Assay

2.7: Host Resistance

2.8: Disease Control.

2.8.1: Cultural Control.

2.8.2: Chemical Control.

2.8.3: Biological Control.

2.8.4: Antagonists.

2.1.1: Taxonomy of causal organism with remarks

Kingdom- Bacteria	
Phyllum- Proteobacteria	
Class- Betaproteobacteria	One of four classes of 'purple bacteria'
Order- Burkholderiales	_
Family- Burkholderieriaceae	Group has nine genera.
Genus- *Ralstonia*	Genus has five species.
Species- *R. solanacearum*	Species has five races. All the five races are plant pathogenic. Some with wide host range and some with narrow host range.
Race 3	Race 3 is found in India and attack tomato plants. The genus was earlier known as *Pseudomonas solanacearum*

Tomato bacterial wilt is caused by *Ralstonia solanacearum*, formerly known as *Pseudomonas solanacearum.* The pathogen has different races, each of them unique and each of them attacking different plants. Tomato bacterial wilt is mostly caused by race 1 strain, which has a wide host range and can survive in the soil for a long period of time. Race 1 strains are highly variable in

their genotype and aggressiveness on tomato. Some highly aggressive strains can cause severe symptoms, even on “resistant” varieties. Fortunately, such strains are not predominant.

Taxonomic groups should follow the natural divisions that are apparent after characterizing the phenotype and genotype of related organisms and determining their relationship to known taxa. Until recently, however, the knowledge necessary to discern natural groups was often unavailable and many groups were incorrectly classified in phylogenetic terms. This was certainly true for the organisms now considered members of the *Burkholderia* group, which were long classed as nonfluorescent *Pseudomonas* species (Anzai *et. al.,*2000;). However, recent genetic analyses have revealed many new relationships and prompted renaming many bacteria in this and other groups (one Web site with official nomenclature is http://www.bacterio.cict.fr/).

2.1.2: Dissemination of *Ralstonia solanacearum*

R. solanacearum has been found to be one of the main constraints in the field of agriculture as it causes wilt disease by blocking the water transportation path i.e. the xylem. A large number of crops and some ornamental plants are being affected globally though bacterial wilt caused by the most devastating phytopathogen called *Ralstonia solanacearum* (Elphinstone, 2005; Hayward, 1994; Fegan and Prior, 2005). *Ralstonia solanacearum* mostly affects solanaceous crops (Alvarez et al. 2008).

R. solanacearum bacterium is transmitted through water and soil, mostly through waterways (Elphinstone, 2005). It colonizes the vascular system after entering the plant through root, resulting in the occurrence of severe symptoms (Hayward, 1992; Kelman, 1953). Disease outbreaks occur due to presence of *R. solanacearum* phylotype (ph) II biovar (bv) 2 in European waterways (Caruso et al., 2005; Van Elsas et al., 2000, 2001). This bacteria can survive for prolonged period in sterile water, deficient of plant materials (Alvarez et al., 2008;

Kelman,1956; Van Elsas et al., 2001; Van Overbeek et al.,2004; Wakimoto et al.,1982).

Starvation survival state is completely different from that of active growth state (Heim et al., 2002). Under prolonged starvation condition, the change of shape of bacteria to round cell and reduction in its size are considered as their strategies to survive in oligotrophic environment (Novitsky and Morita, 1976; Rollins and Colwell, 1986; Ruiz et al., 2001). The induction of viable and non-culturable state named as the VBNC state is one of the adaptation in the oligotrophic state (Oliver, 2005; Roszak and Colwell, 1987). *R. solanacearum* acquires VBNC state after being exposed to copper (Grey and Steck, 2001).

R. solanacearum is a soil borne microbe and transmits through soil-routes. It infects the host plant through their roots (Xue et al., 2020). The invading bacteria causes wilting by multiplying in the xylem and producing huge amount of exopolysaccharides that leads to the blockage in the vessels affecting the water conductance (Saile et al., 1997). Studies have shown that this pathogen can survive in soil for considerable amount of time without any host. There are many theoretical reasons for this bacterial behavior such as association with plant debris or several weed hosts which are symptomless carriers (Genin and Boucher, 2002). Infection in the plants mainly occurs though root-to-root transmission and movement of the contaminated soil (Gopalakrishnan et al., 2014). Swimming motility of *R. solanacearum allow*s it to find suitable host and disperse effectively (Kersten et al., 2001)

The long distance transmission of the pathogen is mainly through infected propagation materials such as potato seed tubers (Abdurahman et al., 2019). The wilt disease cause by *Ralstonia solanacearum* has broad geographical distribution and the dissemination through infected potato seed tubers occurs at a very high rate, contributing to the pathogen dispersal (Elphonstone, 2005; Mansfield et al., 2012; Buddhenhagen 1986; Janse 1996; Janse et al. 2004). *R. solan*acearum lie dormant in propagative organs like

tubers, suckers, seeds or rhizomes and infected plant debris of crops. Infected potato tubers are the main source of dissemination of this pathogen (Choudhary et al., 2018). The widespread of this disease is due to the transmission of these phytopathogen through latently infected ginger rhizome and limited crop rotation due to decreasing land holdings (Kurabachew and Ayana, 2016).

The bacterium can also be transmitted through insects as bacteria can survive on insects for several days resulting in the initiation of crop infection. There is a clear chance of fast disease transmission as insects can move from one plant to another plant within a short period of time. Insects are particularly attracted to plant wounds which form the main infection site (Wolf and Boer, 2007).

Ramirez et al., (2019) reported that the natural spread of most races of *Ralstonia solanacearum* bacteria is slow although he reported an exceptional case where one race that causes Moko disease in banana and has a potential to spread naturally at faster rate. The association of nematode (*Meloidogyne* sp.) and *R. solanacearum* contributes to the spreading of the diseases (Hayward, 1991).

2.1.3: Detection and Identification

Ralstonia solanacearum causes lethal diseases in most of the economically important crop plants thus affecting the agricultural field to a greater extent. So there is a need to characterize this bacterium through detection and identification. Several techniques have been developed so far for the detection of *R. solanacearum* to control bacterial wilt disease (Machmud and Suryadi, 2008).

Grover et al. (2009) performed multiple displacement amplification PCR amplification (MDA-PCR) for detection. Detection and identification are conceptually and methodologically intertwined processes. He performed this

technique on pure cell lysates and soil samples. DNA of pure cell lysates of *R. solanacearum* and soil sample DNA was used as template in MDA reaction. Sample buffer, reaction buffer and enzyme mix were need in MDA procedure. In study of pathobiology and epidemiology of *R. solanacearum,* it is usually necessary to first detect its presence (based on a tentative identification) before isolation and rigorously identifying a strain.

Substantial international effort has been focused on developing better detection methods, because soil and water samples typically have low populations of *R. solanacearum* and not all cells may grow *in vitro* (Anon., 2004; Alvarez, 2005; Denny *et al.*, 2001; Elphinstone *et al.*, 1996; Saddler, 2000; Seal and Elphinstone, 1994). Pure cultures of *R. solanacearum* are not difficult to identify. Cultural and physiological tests can quickly rule out related organisms (Anon., 2004). There are also commercially available fatty acid methyl ester (FAME) analyses and BIOLOGTM kits (Black and Sweetmore, 1993; Janse, 1991; Li and Hayward, 1993; Stead *et al.*, 1992)). Several nucleic acid and serological based methods are also present (Alvarez, 2005; Seal et al., 1994).

One of the effective serological techniques used for detection and identification of bacterial plant pathogen is the Enzyme–Linked Immunosorbent Assay. This technique is rapid, cost–efficient, and practical for field application and does not require sophisticated equipment (Machmud and Suryadi, 2008). In Indonesia, many workers use this technique to detect plant diseases (Machmud et al., 1996). Techniques of ELISA basically involve reaction between antigen (Ag) and antibody (Ab). The technique needs substrates and enzymes to label the reaction that produces colour which can be observed either by our naked eyes or using ELISA Reader (Converso and Martin, 1990; Mc Laughlin and Chen, 1990). The ELISA technique had been modified to increase its effectiveness and had given different names, such as Direct ELISA, Indirect ELISA, Double Antibody Sandwiched ELISA (DAS – ELISA) (Canale et al.,1983 ; Stobbs and Barker ,1985 ; Yadi et al., 1998).

Modification of the ELISA includes the production of Polyclonal antibodies and components of ELISA kits which is needed for the detection of some viral and bacterial plant pathogens including *R solanacearum* (Machmud et al., 1996, 1997, 1998, 1999,).

Lemessa and Zeller (2006) collected eighty one isolates of *Ralstonia solanacearum* like bacteria from different crops like tomato, potato and pepper. Out of those eighty one isolates, sixty two strains were detected and identified as *R. solanacearum* based on tomato pathogenecity bioassay, carbon source utilization patterns and PCR based assay. Out of these 62 strains which have been identified as *R. solanacearum*, 19 were identified as biovar I and 43 strains were identified as biovar II by Hayward's classification method which is based on carbon source utilization patterns. They also performed PCR with the isolates of *R. solanacearum* which produced wilt symptoms in tomato pathogenicity bioassay. They Extracted genomic DNA using the "DNeasy Tissue" Kit and PCR-amplified in a thermal cycler.

Ramirez et al. (2019) have assesed the phylogenetic relationship, diversity and pathogenecity of *R. solanacearum* involved in the production of moko disease in Colombia. They performed multiplex PCR of the 65 isolates obtained from the growing regions of banana / plantain to determine genetic diversity of the isolates. They analysed partial sequences of egl, mutS and rplB genes. From their assessment, they concluded that all the strains belonged to *R. solnacearum* phylotype II, sequevers 4 and 6.

2.1.4: Conditions favourable for development of *Ralstonia solanacearum*

R. solanacearum is an aerobic obligate phytopathogen (Tahat and Sijam, 2010). It survives well in high-drained soil having high moisture content. Its survival in the soil is basically dependent on temperature (Choudhary et al., 2018). Bacterial population has been shown to be reduced when exposed to high day temperature of 40°C for more than four hours (van Elsas et al., 2000). Increase in the disease incidence of bacterial wilt on host, such as tomato and

increase in the ambient temperature were found to be correlated as stated by Hayward, (1991).

Wung and Lin (2005) described the favourable conditions such as high temperature and moist soil are required for the development of bacterial wilt in tomato. They found that the development of bacterial wilt in tomato slowed down on reducing the soil temperature below 20° C or in the reduced soil moisture content.

The pathogen growth is found to be suppressed in some soil types as the soil moisture determines the level of some antagonistic population, which is a competitor of *R. solanacearum*. The association of nematode (*Meloidogyne* sp.) and *R. solanacearum* contributes to the spreading of the diseases (Hayward, 1991). In the hill regions of India, the wilt disease is highly predominant throughout the year where the soil is acidic (Velma et al., 2014)

2.1.5: Distribution and host range of *Ralstonia solanacearum*

The host range of *R. solanacearum* is wide. *R. solanacearum* is an economically important pathogen as it is known to infect large number of crop plants. It causes disease in potato *(Solanum tuberosum)*, tomato (*Lycopersicon esculentum*), peppers (*Capsicum annum*), egg plant (*Solanum melongena*), ginger (*Zingiber officinale*) and a few weed species bitter sweet (*Celastrus orbiculatus)*, stinging nettle (*Urtica dioica*) and night shade (*Solanum karsense*). Earlier strains of *Ralstonia solanacearum* were classified into five races based on the host range. The five races of *R. solanacearum* have different host ranges and wide distribution. Southern United States as well as Africa, Asia and South America are the places where Race I was endemic. Race I was reported to infect a wide range of crop plants. Race 2 was reported to causes wilt disease mainly in bananas and was endemic to the Central America and South East Asia. Race 3 was distributed worldwide and attacks mostly potato. Race 4 infects ginger in Hawaii and Asia. Race 5 is known to infect mulberries in China (Tahat and Sijan, 2010).

Ralstonia solnacearum was divided into six biovars based on its ability to metabolize three diasaccharides and three sugar alcohols (Hayward, 1964; 1991 and 1994).

Bacterial wilt disease caused by *R. solanacearum* generally occurs in lowlands in tropical and subtropical regions but Race 3 biovar 2 (R3bv2), a sub-group of *R. solanacearum* was exceptionally present at high altitudes and temperate regions (Elphinstone, 2005). R3bv2 was known to cause brown rot of potato in highland tropics of Latin America, Africa and Asia (Elphinstone, 2005). It was found that Biovar 3 had the widest host range (Hassan et al., 2016). Chandrashekhar and Prasann, 2010 found two new host plants called davana (*Artemisia pallens*) and coleus (*Coleus forkohlii*) which showed typical wilting symptoms after infection with *R. solanacearum*. Both the crops were important in aromatic and medicinal industries.

2.2.1: Diseases caused by *R. solanacearum*

Gopalkrishnan et.al (2014) Bacterial wilt reduces the production of eggplant in India. The occurrence of the disease has been reported to be much more pronounced in tropical, subtropical and some warm temperate regions of the world. The disease was known to spread fast due to its soil-borne nature and occurs in a wide range of hosts. The disease is transmitted through insects, root-to-root transfer, and dissemination of farm implements and movement of the soil. The conditions like high temperature and poor drainage also trigger the development of the disease resulting in the loss of productivity of crops during summer in India.

Yadessa et.al, (2010) reported that *Ralstonia solanacearum* causes wilt disease in several economically important crops like tomato, potato, egg plant, pepper and tobacco in Ethiopia. Tomato was the most widely grown crops of Ethiopia. Conditions like high temperature, dry days and cooler nights, altitudes between 700 and 2000metres in Ethiopia favoured the production of tomato plants. However, bacterial wilt was the common hindrance in the

production of tomato plants there. Incidence of bacterial wilt in tomato in Ethiopia was very high (55%).

Coutinho et al. (2000) stated that first report of *Ralstonia solanacearum* was found in late 1980s in Brazil. Similarly he also stated that in 1997, bacterial wilt on Eucalyptus in South Africa was found. In the affected plants, the vascular tissue was discoloured and bacterial exudation was produced from cut surfaces.

Mepharishvili et al. (2012) stated that in June, 2010, wilt disease of tomato seedlings in western Georgia was confirmed for the first time. The disease caused drastic loss in the productivity. They also reported presence of infection of some egg plants and sweet pepper plants in some *R. solanacearum* infected regions. The symptoms like wilting and vascular tissue discolouration were observed.

2.2.2: Fusarium Wilt

The other most economically important pathogen group is *Fusarium oxysporum* species which comprises a group of strains that are known to cause vascular wilt disease known as *Fusarium* wilt in most of the economically important crops throughout the world. Variation in the pathogen is due to the horizontal gene transfer. Sexual reproduction is not known in this pathogen. Development of the disease depends on several factors like the structure of the root cortex, capacity of the host to recognize and response to the growth of the pathogen and composition of root exudates (Gordon, 2017).

Fusarium wilt is one of the most devastating diseases in plants that cause huge loss to the agriculture. The causal organism of this disease is *Fusarium oxysporium or Fusarium solani.* Tomato plants are mostly affected by this disease thoughout the world, especially in the upland areas. More than 100 *Fusarium* vascular wilt diseases have been reported worldwide (Ajilogba and Babalola, 2013).

Fusarium wilt was first reported in India by Butler in 1918 and Later Padwick in 1940 correctly determined its etiology (Jimenez-Diaz et. al., 2015). They also reported Chickpea had a significant role in farming system. But *Fusarium* wilt was the main hindrance in the path of production of chickpea. *Fusarium* wilt was one of the most economically important diseases affecting chickpea worldwide. Symptoms of the disease appear at any stage of plant growth. The infected plants formed group as patches. Symptoms were flaccidity of individual leaves, dessication, dull green discolouration, collapse of the whole plant. Fusarium wilt reduced chickpea production by reducing both seed weight and seed yield.

2.3: Loss caused by *Ralstonia solanacearum*

Knap et al. (2004) reported that the bacterial wilt was one of the most destructive plant diseases affecting most of the economically important crops including plants of solanaceae as main target of the pathogen in Ethiopia. In the solanaceae family the most affected plants were tomato, potato, eggplant and pepper. They also reported that those crops were economically important as Ethiopian farming community primarily depended on those crops as sources of their income and food security. They reported that substantial yield loss of different solanaceous crops by the bacteria were found to occur from time to time in different parts of the country.

Seleim et al. (2014) isolated and identified fifteen *Ralstonia solanacearum* isolates from Egypt and confirmed their pathogenicity which showed that all isolates were pathogenic to tomato plants and their wilting capacity varied from 52 to 97%. They found whitish ooze from the cut stems of symptomatic plants when submerged in water.

Chilli pepper (*Capsicum annum)* is one of the most important vegetables from the family solanaceae because of its use as spices and condiments (Aslam et al., 2017). Bacterium *Ralstonia sola*nacearum is one of the most economically important parasite that cause huge loss in the yield of Chilli

pepper (Elphinstone 2005; Wicker et al. 2007). It had a devastating effect on crops worldwide especially in the tropical regions (Artal et al. 2012).

2.4: Symptoms caused by *Ralstonia solanacearum* in tomato and allied crops:

Symptoms of the wilt disease appear after few days of infection (Buddenhagen and Kelman, 1964; Haywards, 2000). *R. solanacearum* makes its way to the plant through roots having wounds that are made by insects or certain nematodes, transplanting, cultivation or natural wounds where the secondary root arises (McCarter, 1991). After entering inside the host, the bacterium establishes itself in the vascular tissue, where it multiplies rapidly, blocking the water transportation, which causes wilting (Hayward, 1991; Wang and lin, 2005). Symptoms, such as, wilting, dwarfing, later yellowing is observed. Death of the plant occurs due to the filling of host cell with bacterial cells and exopolysaccharaide slime (Buddenhagen and Kelman, 1964; Hayward, 2000). Later, discolouration of the vascular system from pale yellow to dark brown occurs. Further symptoms represent exudation of droplets of milky bacterial ooze from infected tissue (McCarter, 1991).

Infection of the host plant by *R. solanacearum* may express all or none of the symptoms. This observed condition is known as Latency where the plant infected with pathogen expresses all or none of the symptoms, even under ideal conditions (Tahat and Sijam, 2010).

There may be some variation in the symptoms of *R. solanacearum* among the wide range of host (Garcia et al., 2018). The wilt is located on one side of the plant i.e. unilateral (Denny, 2006)

Younger tobacco (*Nicotiana tabacum*) leaves are the first to get wilted at the early stages of infection (Denny 2006; Echandi ,1991). In the next stage, the leaves turn yellow and followed by scorch –like symptoms between the veins and at the margins. Total collapse of the plant occurs at the later stages

(Denny, 2006). Echandi, 1991 reported that plants that do not die show leaf distortion and stunting like symptoms. Primary and secondary roots may rot.

Wilting occurs specifically on the younger leaves of tomato (Momol and Champoiseau ,2009). The first visible symptoms are chlorosis and wilting located at the tip of branches of potato (Denny 2006 ; Stevenson et al.,2001). Petiole epinasty is the another symptom that occur on potato (Denny 2006; Stevenson et al.,2001)

In June, 2010, farmers in Chkhorotsku region, Western Georgia reported wilt disease infecting tomato plants, causing plant loss up to 100%. Farmers in Kutaisi region also observed similar symptoms of bacterial wilt, such as, wilting and discolouration in tomato plants (Mepharishvili et al., 2012).

Depending upon the diverse nature of the pathogen infecting various host plant, there exist several bacterial wilt symptoms. some bacterial wilt symptoms occur as flabby appeareance on the youngest leaves of tomato, usually at the hottest time of the day, few days after inoculation (Vasse et al.,1995).

2.5: Virulence, pathogenesis and Fli C gene

Virulence is a special ability of an organism to cause disease by involving some virulence factors. These factors are either cytosolic, membrane associated or secreatory in nature. There are several regulatory pathways that control the expression of several virulence factors in *Ralstonia solanacearum* (Brito et al., 2005).

Trans-Kerstein et al. (2001) reported that *R. solanacearum* in planta was essentially nonmotile, but it was highly motile in culture. To determine the role of pathogen motility in this disease, they cloned, characterized, and mutated two genes in the *R. solanacearum* flagellar biosynthetic pathway. The genes for flagellin, the subunit of the flagellar filament (*fliC*), and for the flagellar motor switch protein (*fliM*) were isolated based on their resemblance to these proteins

in other bacteria. As is typical for flagellins, the predicted FliC protein had well-conserved N- and C-terminal regions, separated by a divergent central domain. The predicted *R. solanacearum* FliM closely resembled motor switch proteins from other proteobacteria.

He et al. (2012) reported FliC, as a flagellar filament structural protein, and hypothesized to be involved in the pathogenesis of infection in case of *Edwardsiella tarda*, a flagellated Gram-negative bacterium which causes edwardsiellosis in fish. They studied, a *fliC* in-frame deletion mutant of a virulent isolate of *E. tarda* was constructed through double crossover allelic exchange by means of the suicide vector pRE112, and its virulence-associated phenotypes and pathogenicity were tested. It was found that the deletion of *fliC* significantly decreased the diameter of flagella filaments. In addition, the mutant showed reduced pathogenicity as well as showed impaired bacterial growth, reduced motility, decreased biofilm formation and reduced levels of virulence-associated protein secretion involved in the type III secretion system (TTSS). The phenotypic characteristics of the *fliC* deletion mutant uncovered in this investigation suggest that *fliC* plays an essential role in normal flagellum function, bacterial growth, protein secretion by TTSS and bacterial virulence.

Haiko and Westerlund-Wikstrom (2013) stated that flagellin or FliC, The major subunit, of the flagellum plays a well-documented role in innate immunity and as a dominant antigen of the adaptive immune response. Importantly, flagella have also been reported to function as adhesins. Whole flagella have been indicated as significant in bacterial adhesion to and invasion into host cells. In various pathogens, e.g., *Escherichia coli*, *Pseudomonas aeruginosa* and *Clostridium difficile*, flagellin and/or the distally located flagellar cap protein have been reported to function as adhesins. They also reported that FliC of Shiga-toxigenic *E. coli* was shown to be involved in cellular invasion via lipid rafts. They studied flagellar adhesive and invasive properties, especially focusing on the flagellum as a potential virulence factor.

Tans-Kersten et al (2004) reported that motility was a virulence trait of *Ralstonia solanacearum* and they showed that motility allows the bacterial wilt pathogen *Ralstonia solanacearum* to efficiently invade and colonize host plants. They showed in culture, flhDC expression depended on PehSR, a regulator of early virulence factors; and, in turn, FlhDC was required for fliC (flagellin) expression. They also showed that fliC gene was expressed in planta at cell densities where motile bacteria were not observed, as well as in a nonmotile flhDC mutant. Thus, expression of flhDC and flagellin itself are uncoupled from bacterial motility in the host environment, indicating that additional signals and regulatory circuits repress motility during plant pathogenesis.

Brito et al., (2005) reported that PhcA is a part of regulatory network that express several virulence factors. It controls several virulence factors either directly or indirectly (via some intermediatory regulatory genes). PhcA is involved in the bacterial motility, plant cell wall degrading enzymes and expression of extracellular polysaccharide (EPS) (Huang et al.1995).

Pathogenicity of *R. solanacearum* is also determined by hrp-encoded Type III secretion system (TISS). TISS translocate effector proteins into plant dells (Genin et Boucher, 2004). Disease development occurs due to *hrp* gene. For induction of a defensive hypersensitive response (HR), gene *hrp* is required. Once pathogen gets recognized, this hypersensitive response is triggered. Growth environment strongly influences the *hrp* gene expression in several Gram–negative bacteria (Arlat, 1992; Huynh et al. 1989).

Many cellular processes like virulence, stress tolerance etc. are related to cold shock proteins (Liu et al., 2020). Cold–shock proteins (Csp) are small and highly conserved proteins. They comprises cold–shock domain, the RNA binding domain (Timonon et al., 2016; Chaikam et al., 2010; Eshwar et al., 2017). Research on the Csps of *R. solanacearum* is scanty. Many works based on the genomic data have been done to know the role of Csps in virulence of *R. solanacearum*. The role of HrpG and XpsR have been correlated with the host-

bacterium interactions and expression of extracellular polysaccharide (EPS) related genes (Genin, 2010; Genin et Denny, 2012).

2.6: Host Resistance

The most effective disease control method is producing resistant cultivars against bacterial wilt. This is considered as the most economical, eco- friendly method of disease control. The most economically important crops such as potato, tomato, pepper, peanut, tobacco and eggplant have been selected for developing resistant cultivars against bacterial wilt disease though breeding (Boshou, 2005). Some factors such as temperature, soil moisture, pathogen strains, host–pathogen interaction, presence of root-knot nematodes, genetic linkage between resistance and breeding methodology highly control the stability of resistant varieties (Boshou, 2005; Elphinstone 2005; Wang and Lin, 2005).

Getachew et al. (2009) assessed all the tomato plants affected by the disastrous strain of *R. solanacearum* originated from Ethiopia. They found that six were resistant, eleven moderately resistant whereas most of the genotypes including all tomato cultivars commonly grown in Ethiopia were found highly susceptible. Colonization and invasion of *R. solanacearum* on resistant plants makes them more tolerant to diseases. Nakaho et al. (2004) reported that multiplication of bacteria was suppressed in the stems of resistant tomato plants due to restricted pathogen movement between the xylem tissues basically from the protoxylem to other xylem tissues. Furthermore, Dahal et al. (2010) used proteomic approach to explain the molecular interactions in the cell walls of resistant and susceptible plants infected with *R. solanacearum*.

2.7: Disease control

R. solanacearum causes huge yield loss on different economically important crops worldwide (Kurabachew and Ayana, 2016). It causes 50-100% loss in potato yield in Kenya (Muthoni et al.,2012), 70% on potato in India

(APS, 2005) and 88% on tomato in Uganda (Kataflire et al., 2005). It causes considerable economic loss every year (Elphinstone, 2005). Thus, control of the disease is required to minimize crop loss (USDA, 2003). Management of bacterial wilt disease is really difficult because of its wide host range, long survival in sterile water and soil, several ways of transmission, genetically diverse strains (APS, 2005; EU, 2003). There is a need to standarise different management programs to control bacterial wilt disease (Kurabachew and Ayana, 2016). Collective application of all management programs is needed (Lemessa and Zeller ,2007; Bekele and Berga, 2001). Different methods such as Agronomic practices (Bekele and Berga, 2001; Janiver et al., 2007), host resistance (Boshou, 2005), chemical control (Fortnum and Martin, 1998; Santos et al., 2006; Edward Jones, 2008). Biological control (Bias, 2004; Whips, 2001; Whipps et al., 2007; Kurabachew et al., 2007; Lemessa and Zeller, 2007; Alyie et al.,2008; Kurabachew and Wydra, 2013; Ciampipanno et al.,1989 and Intregated disease management (Yuliar et al., 2015; Kinyua et al. 2001).

2.7.1: Agronomic practices

Cultural practices, if used properly, can efficiently decrease the incidence and severity of Bacterial wilt disease. Soil borne bacterial population can be reduced by the application of crop rotation with nonsusceptible crops (Bekele and Berga, 2001). Identification and use of “non-host break crops” and “exact rotation period” should be taken into consideration. Plantation time should be shifted to cooler periods of the year to escape the disease. Particular phyto-pathogenic population is established on continuous cropping with same susceptible host, therefore crop rotation with change in varieties is also need to be considered to reduce the disastrous effect of soil–borne pathogen (Janiver et al., 2007). Katafire et al., (2005) discovered that the wilt disease incidence was reduced by 64% to 94%, after rotating cultivation of potato with sorghum, millet, sweet potato, wheat, carrots or phaseolus beans.

Soil amendment has also been used in many areas to control bacterial wilt disease and was found to restrict the growth of pathogens in the soil (Michel and Mew, 1998). Application of organic amendments to soil improvises the chemical, physical and biological properties of soil, which directly increases crop productivity (Bailey at al., 2003). Survival of the pathogen is highly affected by the degradation of organic matter present in the soil, which restricts available nutrients in the soil (Bailey et al., 2003). Degradation of organic matter releases inhibitory chemical substances that enhances the activity of antagonistic pathogen in the soil (Bailey et al., 2003; Akathar and Malik, 2000).

Lemaga et al. (2001) reported that application of organic materials (*Leucaena diversifolia* and *Sesbania sesbana*) in soil either singly or combined with inorganic fertilizer decreased wilt disease incidence, thus yield of potato tuber was increased.

Getachew et al. (2011) reported that the combined effect of silicon fertilizer and sugarcane bagasse (alternatively used silicon source) on wilt incidence and reported that tomato fruit yield was increased.

Bacterial wilt incidence could be reduced significantly by 81% by the application of soil amendments combined with coco peat farmyard manure (FYM) compost in the soil (Yedessa et al., 2010)

Lemaga et al. (2005) reported that the bacterial wilt was reduced by 29% and 50% on the application of Nitrogen(N) + Phosphorus(P) + Potassium(K) and Nitrogen(N) + Phosphorous(P) respectively.

2.7.2: Chemical control

Several fumigants such as metal sodium, 1,3-dichloropropene and chloropicrin and pesticides such as 3-[3 –Indolyl] butanoic acid have been worked efficiently against bacterial wilt disease. Several plant activators, such as, Validamycin A and Validoxylamine, which trigger plant systemic resistance have been used in tomato to control bacterial wilt disease. The combination of

metal sodium with choloropicrin significantly reduced the disease incidence from 72% to 100% and increased tobacco and tomato yield in the field. The yield of tomato was 1.7 to 2.5 fold higher when treated with pesticides (Fortnum and Martin, 1998, Santos et al., 2006).

Edward–Jones (2008) reported that pesticide offered a significant reduction in the bacterial wilt disease in many fields, but its effect on plants may differ depending on the way it has been used. If farmers use pesticides without proper knowledge and care, some amount of it may remain in the soil for many years (Gadeva and Dimitrov, 2008) ,become contaminated in groundwater and soil (Acero et al.,2008) and become poisonous (Dasgupta et al., 2007).

Chlorine dioxide (ClO_2), Chloro –oxide (CaClO) and organic acids such as acetic acids and lactic acids are effective in controlling bacterial pathogens (Janse ,2002; Choudhary et al., 2018).

Pradhanang et al. (2002) reported the use of essential oils as soil fumigants to control bacterial wilt disease. Treatments with thymol, lemongrass oil and palmarosa oil gave positive results on the control of the *R.solanacearum* population. The fungicidal effect of essential oils extracted from medicinal plants is well known (Wilson et al., 1997).

Systemic resistance is induced by the application of Acibenzolar –S-methyl (Hacisalihoglu et al., 2007; Pradhanang et al., 2005; Yuliar et al., 2014). The combination of thymol and Acibenzolar – S- methyl (ASM) suppressed the disease incidence and increased tomato yield, but thymol and ASM alone could not do the same (Hong et al., 1996)

Silicon (Dannon and Wydra, 2004; Kurabachew and Wydra, 2014; Wydra and Dannon, 2006) and Chitosan (Kirkegaard et al., 1996) are known to induce induced resistance , thus helps in the reduction of the incidence of the bacterial wilt disease

Bacteriostatic action with Phosphoric acid solution can be used to control infection caused by the bacterial wilt pathogen (Norman et al.,2006).

The use of antibiotics such as streptomycin increased the bacterial wilt in Egypt, thus questioned the application of antibiotic in controlling plant diseases as it is responsible for the development of resistant strains of bacteria (Farag et al., 1986; OEPP/EPPO, 2004).

Amzalek and Cohen (2007) examined the efficacy of four inducers (AABA, BABA, GABA AND NaSA) to control rust infection in sunflower plants caused by *Puccinia helianthi* . BABA was found to be the most effective inducer and sodium salicylate (NaSA) was the least effective in controlling rust infection caused by *Puccinia helianthi* in sunflower plants.

SA, AABA, GABA, BABA and BTH are known to act as elicitors which has the capacity to induce natural defense system of plants (Gomez –Vasquez et al., 2004). Silue et al.,(2002) reported the efficacy of BABA as inducer in inducing defensive response in Couliflower (*B. oleracea* var.botrytis) against *Peronospora parasitica*, the causal agent of downy mildew.

Sharma and Sohal (2016) reported the effects of GABA in inducing defensive system in Indian mustard (*Brassica juncea* var. RLM619). GABA may help in triggering the level of antioxidant content in Indian mustard, thus conferring its resistance against various fungal diseases (Sharma and Sohal, 2016).

2.7.3: Biological Control

The use of biological control plant disease has been increased in recent times due to public concerns about the effect of using chemicals in the fields (Whips, 2001). The reason behind this is the self –sustaining nature of biological control agents (Whipps et al., 2007). This are many other promising characteristics of the biological control agent's such as: long-term disease management in an eco- friendly manner, potentially spread on their own after

initial establishment, reduced use of nonrenewable resources (Whipps et al., 2007).

The rhizosphere resident microbial antagonist is considered as a favourable approach in the management of several plant diseases. The rhizosphere is a place where several biologically important processes and interactions occurs (Bias, 2004). Several studies have shown that the use of various antagonistic species have helped in controlling bacterial wilt. Major antagonistic species are *Bacillus subtilis, B. cereus, B. pumilis, Pseudomonas putida, P. fluorescens, Paenibacillus macerans* and *Serratia marcescens.* These were collected from potato and tomato rhizosphere from Ethiopia (Kurabachew et al.2007, Kurabachew and Wydra, 2013; Lemessa and Zeller, 2007; Alyie et al., 2008). Ciampi Panno et al. (1989) have proved the role of antagonistic bacteria to manage *R. solanacearum* population under field condition.

Potential biological agents include genetically engineered antagonistic bacteria (Kang et al., 1995), avirulent mutant of *R. solanacearum* (Dong et al., 1999) and some naturally occurring antagonistic rhizobacteria such as *Bacillus subtilis*, *B. cereus*, *B. pumilis, Pseudomonas fluorescens, P. putida, Serratia marcescens* and *Paenibacillus macerans* (Alyie et al., 2008; Lemessa and Zeller, 2007; Kurabachew et al., 2007; Kurabachew and Wydra, 2013).

Chapter-3

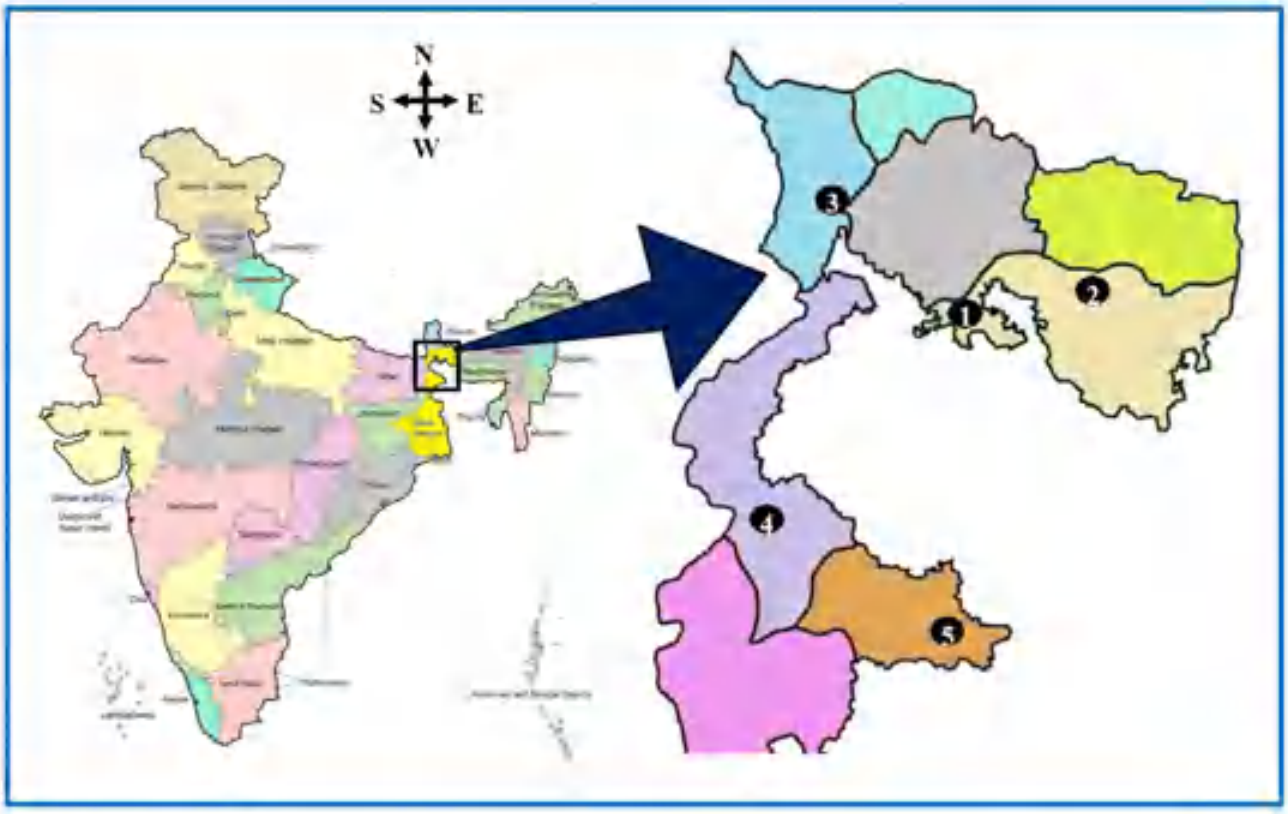
MATERIALS AND METHODS

3.1: Survey of different tomato growing fields of North Bengal, sample collection and selection of sample collection fields:

Roving survey was carried out in the different tomato growing fields of North Bengal to identify the affected areas (Fig.3.1). Severely infected areas were identified and affected plants were collected. Soil samples were collected from the rhizosphere of the infected plants and were brought to the laboratory of Department of Botany, University of North Bengal for further experiments. Wilted tomato plants were collected and were brought to the laboratory. Fifty different collection points were identified from where diseased plants were collected. Out of the 50 different collection points 26 points were found to be bacterial wilt prone area. The samples from these 26 points were subjected to bacterial isolation by ooze collection procedure. Thus all the 26 bacteria isolated were subjected to preliminary identification by morphology and biochemical characteristics. However, the most virulent isolates were also identified by molecular 16S rRNA studies.

3.2: Host plants:

One local variety and four certified tomato seed varieties (PKM-1, Vaishali, Rupali and Rashmi) were used during the present study. All the five varieties were selected for plantation in the experimental garden, Department of Botany, University of North Bengal. The four certified varieties were selected on the basis of their availability in the local seed shops and also for their popularity among the local farmers. On the basis of verbal discussion with the local farmers it was known that they preferred the varieties on the basis of their satisfactory growth in the agro-climatic condition of North Bengal. Excluding tomato plants, the brinjal and potato plants were also used for the host range study. The varieties of brinjal potato and Chilli were 'Muktakesi', 'Kufri jyoti' and 'Kull Sona" respectively. About 10 cm long healthy plants were collected from farmers' fields of Phansidewa (District Darjeeling)



1. Haldibari[26.33°N88.77°E](Coochbehar)
2. Ghoshkadanga[26.43°N89.27°E](Coochbehar)
3. Siliguri[26.71°N88.43°E](Darjeeling)
4. Durgapur[25.52°N88.15°E](UttarDinajpur)
5. Balurghat[25.24°N88.79°E](DakshinDinajpur)

Figure:3.1:Mapshowingplacesofdiseasedsamplecollection.Nameinparenthesesa rediffferentdistricts ofsub-Himalayan,WestBengal.

3.3: Plant germination and maintenance:

Tomato seeds of the selected varieties were surface sterilized with 0.1% mercuric chloride solution for 1 min and then washed thrice with sterile distilled water. Seeds were dipped in seed-bed (earthen tray or directly on soil) for germination. Tomato Seedlings about 10 cm long, from the earthen tray were finally transferred to earthen pots (16 cm in diameter and 12 cm in height) containing soil-farm yard manure mixture of 200g of farm yard manure and 1800g of fine soil. The same mixture was also prepared and used for plants transferred to plots. One plant was grown in one pot. In the plots plants were sown in a row in such a manner so that distance between plant to plant and row to row was 45 cm (Fig. 3.2).

Other plants (Potato, Brinjal and Chilli) were also transferred to experimental pots/plots directly from farmer's fields. The plants were maintained in the garden at natural temperature (25^0C-30^0C) and light; pH of soil was about 5.6 to 6.0. Plants were watered as and when required. The source of water was natural tap water. Weeds were removed regularly.

3.4: Bacterial pathogens/antagonist: The infected plant samples and rhizosphere soil samples were collected and brought to the laboratory for the isolation of the bacteria through bacterial ooze collection (in case of pathogen) and by serial dilution technique as suggested by Dhingra and Sinclair (1995) in case of isolation of antagonistic soil bacteria.

3.4.1: Isolation of pathogenic bacteria isolates from infected plants: All plant samples were washed with sterile distilled water and surface of the crown region was surface sterilized with 70% alcohol. Finally, a cross section of the plant stem was made with a sharp sterilized blade. The root portion was removed and the cut end of the stem was immediately introduced in to a tube containing sterile distilled water. A stream of white, milky layer of bacterial cells (bacterial ooze) in clear sterile distilled water was observed. Then the water was used as sample for isolation of bacteria as suggested by Leppla *et al.*

(2004). Bacterial suspension (0.1 ml) was spread on *Pseudomonas solanacearum* agar medium and incubated at 32° C for 24 hours.

Fig. 3.2: a) Tomato plants in the experimental garden of Department of Botany; b) Potted plants in the experimental garden

3.4.2: Isolation of antagonistic bacteria from soil:

The rhizosphere soil samples were semi dried in laboratory and then 10 gram soil was mixed in 100 ml of sterile distilled water. The resultant solution was considered as stock solution. From the stock solution serial dilution series were made up to the dilution of 10^{-5}. The 10^{-4} dilution has given the ideal plate count (30 cfu to 300 cfu per ml) count. One ml of this dilution was allowed to grow in 20 ml Nutrient Agar medium supplemented with 2mg Nystatin (Fungi static compound). After 72 hours of incubation at 30°C, a large bacterial colony intermingled with several other colonies. If any bacterium found to show antagonistic behaviour against other bacteria nearby in the plate, then that bacterium was picked up by sterile tooth-pick and plated in a sterile Nutrient Agar medium. Thus antagonistic bacteria were isolated and purified.

In the present study one fungal antagonist *Trichoderma harzianum* was used which was procured from Prof. Aniruddha Saha of Molecular Plant pathology Laboratory of Department of Botany, Universiyty of North Bengal. The fungal culture was originally collected from Indian Agricultural research Institute, New Delhi (ITCC-4572).

3.5: Pathogenicity test:

Pathogenicity test was performed in potted plants as well as in field grown tomato plants.

3.5.1: Pathogenicity test in potted plants and verification of Koch's postulations:

Bacterial pathogens of the present study were subjected to pathogenicity test following the method as suggested by Hoque and Mansfield, 2005. Healthy (10 cm long) tomato plants [Local cultivated variety] were transplanted into earthen pots (size: 16 cm diameter × 12 cm in height) containing sterilized garden soil. All the bacterial isolates to be tested were freshly cultured in nutrient broth medium. Ten ml of 72 h old broth was diluted to 50 ml by

sterilized distilled water. The diluted culture was mixed with the soil surrounding the test plant. In case of control 50 ml of sterilized distilled water was added instead of diluted culture. Incidence of *Ralstonia wilt disease* was evaluated up to 15 days at 3 days intervals and was recorded as days after pathogen inoculation (dapi). Severity of the symptoms was graded into five disease classes as suggested by Kobriger et al., (1998) with minor modifications. Wilting index was graded as follows: + = up to 20% wilted; ++ = 20-40% wilted; +++ = 40-60% wilted and++++ = 60-80% wilted and +++++= 80-100% wilted. Ten plants were taken for each treatment. Average disease development of ten plants of each treatment was considered as wilting index to evaluate disease development.

Koch's postulations were verified in order to ascertain the isolated bacterium is a pathogen or not. For this, each bacterium, isolated from bacterial ooze was tested for its ability to induce wilt disease in tomato plants grown in pots and those have proved potential as pathogen in 'pathogenicity test' were subjected to re-isolation of the bacteria from the cut end of the stem as mentioned earlier. The re-isolated bacterium of each such isolation was biochemically identified as *Ralstonia solanacearum*.

3.5.2: Pathogenicity test in field grown whole plants and disease assessement in susceptible variety:

Soil was ploughed and solarized for seven days thereafter healthy tomato plants (10-12 cm long) of variety PKM-1 were sown in the experimental garden. Distance between plant to plant and row to row was 45 cm. The plants were maintained by natural tap water. Bacterial isolates to be tested were freshly cultured in nutrient broth medium. Ten ml of 72 h old broth was diluted to 50 ml by sterilized distilled water. The diluted culture was mixed with the soil surrounding roots of the test plants. In case of control 50 ml of sterilized distilled water was added instead of diluted culture. Control plants and

experimental plants were kept at 4 meter distance (Fig. 3.3). Incidence of disease was evaluated as described in section 3.5.1.

3.6: Morphological and Biochemical characterization of 26 bacterial isolates:

The classical approach to bacterial identification involves preliminarily microscopic examination following Gram reaction which divides bacteria into two broad groups (Gram + ve and Gram – ve). In addition several biochemical tests were performed to identify the bacteria up to the genus level as suggested by Trigiano *et al.* (2004). The major morphological and biochemical experiments performed were motility test, Gram staining, suitable growth condition (aerobic/anaerobic) on common laboratory media (Nutrient agar & nutrient broth), Yellow pigmentation on YDC medium, Growth on D1M medium, Growth above 40°C, Growth bellow 4°C, Oxidase test. Finally different carbohydrate utilization test with KB009 HiCarbohydrate™ Kit were done for some selected bacterial pathogens.

3.6.1: Gram staining:

Test bacterium was grown in Nutrient Agar slant for 24 hour. Loop full of bacteria was placed on a clean grease free slide and smear was made. The smears were dried in room temperature and air dried smear was heat-fixed. Then the slides were placed in a tray and flooded with crystal violet [composition: 2g Crystal violet, 20 ml alcohol (95%), 80 ml Ammonium oxalate solution(1%)]. Stained for 1 minute and washed in running tap water for 3 seconds. Then the slide was flooded with Burke's Iodine [1 g Iodine, 2g Potassium Iodide, and distilled water 1000 ml] and allowed to stain for 1 minute. Slides were washed with 95% ethanol, which was poured drop by drop holding the slides in slanting condition against a white background till no colour came out from the lower portion of the slide. Thereafter, the saffranin solution was poured on the smears. After 1 minute of staining the slide was

rinsed with water, air dried and was ready for light microscopic study. (Bartholomew, 1962)

Fig. 3.3: Pathogencity test: a) Healthy polant (uninoculated) b)Plant .showing wilt symptom following inoculation by *Ralstonia solanaccarum* (isolate RSG-01)

3.6.2: Aerobic/anaerobic growth study:

Ralstonia solanacearum isolates were grown in thioglycolate broth. Where aerobic bacteria cannot grow and anaerobic bacteria grow at the base because anaerobes are poisoned by oxygen as the concentration of oxygen is lowest at the base. Sodium thioglycolate in the broth consumes oxygen and allows the growth of obligate anaerobes.

3.6.3: Yellow Pigmentation study in YDC medium:

After preparation of YDC medium plates, the plates were inoculated by all the 26 *Ralstonia solanacearum* isolates. If yellow pigmentation was found, then that bacteria were marked as YDC positive.

- **Composition and preparation of YDC medium:**

Yeast extract	10 g
Dextrose	20 g
$CaCO_3$ (light powder)	20 g
Agar agar	20 g
Distilled water	1000ml

Dextrose was separately autoclaved at 15 lb p.s.i. for 15 minutes at 121°C in half of the distilled water and other ingredients were also autoclaved in the other half of the distilled water at 15 lb p.s.i. for 15 minutes at 121°C. Finally solutions were combined and stirred before pouring plates on that CaCO3 was evenly distributed (Schaad, 1988).

3.6.4: Growth on D1M Medium:

Growth on D1M medium was studied for all the 26 bacteria. Fresh D1M plates were inoculated by all the 26 pathogenic bacteria. Bacterial plates were

incubated at 30°C. If any growth was observed visually, then it was recorded as positive. If no growth was found then it was recorded as negative.

- ***Composition and preparation of D1M Medium*:**

Cellobiose	5.0g
NH_4Cl	1.0g
NaH_2PO_4	1.0g
KH2PO4	1.0g
MgSO4, 7H2O	3.0g
Malachite green	3.0 g
Agar agar	15g
Distilled water	1000ml

All the ingredients were mixed at proper ratio and pH was adjusted at 7.0 and then autoclaved at 15 lb p.s.i. for 15 minutes at 121°C. After autoclave the media was cooled at 50 to 55°C and then poured into Petri plates (Schaad, 2001).

3.6.5: Oxidase test:

This test was performed to identify the suspected isolates containing the Cytochrome Oxidase which reduces the electrons of electron transport chain of the isolates to water. When the electron donor was oxidized by Cytochrome Oxidase it turns a dark purple colour which was confirmed the positive test. Bacterial cultures (24 h old) were smeared across a filter paper moistened with freshly prepared 1% tetramethyl-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 seconds indicated a positive reaction (Cowan and Steel, 1993).

3.6.6: Catalase test:

The test organisms were inoculated in a nutrient agar plate. the plate was incubated in a slop for 24 hour. One ml of Hydrogen peroxide solution (3%) was added down the slop. Immediately if gas evolved then that is the indication of catalase activity (Cowan and Steel, 1993).

3.6.7: Carbohydrate utilization test:

For the carbohydrate utilization test the KB009 HiCarbohydrate™ Kit of HIMEDIA was used to study a biochemical profile of the suspected isolates. In this kit the tests are based on the principle of pH change and substrate utilization. After incubation, the metabolic changes were occurred of the isolates and it was indicated by the spontaneous colour change in the media. The kit was divided into three parts like Part A (KB009A), Part B1 (KB009B1) and Part C (KB009C). The pure cultures were used for the test. Homogenous suspension was prepared for the individual isolates with sterile saline and the density of the suspension was 0.5 OD at 620nm. 50µl inoculum was added by surface inoculation method for each selected isolate and incubated at 35°C for 24 to 48 hours.

3.6.8: Growth Below 4ºC and above 40ºC:

Each test bacterium (pathogenic or antagonistic) was freshly streaked on three Nutrient agar medium plates. In case of pathogenic bacteria one plate was incubated at 4°C, one plate was incubated 40°C and the third plate was incubated at 30°C (Control). Growth of the bacterial streak was observed against the control bacterial plate kept at 30°C. Plates were observed up to 72 h. If no growth was found on the basis of visual observation the bacterium growth was recorded as negative. The antagonists grown in nutrient agar medium were incubated at 25°C, 30°C, 37°C and 42°C. Plates were observed up to 72°C.

3.6.9: Host range test of bacterial pathogen:

Generally bacteria survive in a wide host range and cause disease in most preferable target host. The host range test of *Ralstonia solanacearum* was performed in three different cultivated plants belong the Solanaceae family. The test was performed by inoculation of the *Ralstonia solanacearum* in soil of experimental pots as mentioned in case of pathogenicity test. The symptoms were observed for a period of time up to 15 days at 3 days intervals. The hosts which showed symptoms were recorded.

3.7: Morphological study:

3.7.1: Shape of the cells:

To examine the shape and size of cells, a drop of cell suspension of the test organism was placed on a clean grease-free slide, air dried and stained with 10 times diluted carbol fuchsin (phenol 85g, basic fuchsin 15 g, ethanol 250 ml, distilled water 1250ml) and was observed under microscope. Diameter was measured with a standard occular micrometer.

3.7.2: Motility test:

Motility of the isolated bacteria was detected in semisolid motility medium (Barow and Feltham, 1993). Stab containing motility medium was inoculated by a straight inoculating needle. The tubes were incubated at 30°C for 48h and observed for diffused growth around the line of inoculation which indicate positive motility of the test bacteria.

- **Modified motility medium (Hajna, 1950) composition & procedure:**

Peptone	10g
Beef extract	3g
NaCl	5g
Agar	4g
Gelatin	80g
Cystein	0.2g

Ferrous ammonium sulphate	0.2g
Sodium citrate	2g
Distilled water	1000ml

Gelatin was soaked in water for 30 minute. Other ingredients were added and heated to dissolve. Finally, the medium was sterilized at 15lb pressure for 20 minutes.

3.7.3: Light Microscopy:

Gram stained smears on slides as described in section 3.6.1 was observed under light microscope at 40x (Leica Application Suite V4.4 microscope equipped with Leica MC 120 HD digital camera, Singapore). The bacteria tested were determined whether it was Gram positive or Gram negative. Gram negative bacteria show pink or light red colour whereas Gram positive bacteria show blue colour under light microscope.

3.7.4: Scanning Electron Microscopy:

One sterile cover glass was placed in a sterile Petriplate and a bacterial smear was prepared on that coverglass. The cover glass was subjected to series of treatment prior to observation underscanning electron microscope (SEM). The process of Samaranayake *et al.* (2005) was followed for treating the bacterial smear on cover glass. The bacteria were fixed with 2.5% glutaraldehyde solution for one hour. Glutaraldehyde was removed by decanting. Then dehydration of the material was done by an ascending series of ethanol. After dehydration the samples were coated with gold (IB2-ion coater, Japan) and observed under scanning electron microscope [Model: Hitachi S-530(Japan) 1986].

3.8. Studies of bacterial genes for identification:

The isolated bacteria were then identified by molecular techniques using polymerase chain reaction. Gene sequences that included 16S rRNA were analyzed. Molecular detection is the most specific technique for identification of the isolated bacteria. It is also required for better understanding of the phylogeny of the isolated bacteria. In addition, another gene sequence of *fliC* gene was also used for confirmation of the isolates following BLASTn analysis.

3.8.1: DNA isolation:

In this study the bacterial genomic DNA were isolated following the process of Ausubel *et al.*, (1992). At the beginning of the DNA isolation process the Nutrient Broth culture media was prepared for the preparation of fresh bacterial culture. Incubation of 1 cfu in 5 ml of Nutrient Broth was done at 30°C for overnight at 120 rpm. After getting the sufficient growth the cell suspension was centrifuged for 2 min at 13,000 x g. Then the supernatant was discarded and the pellet was first dry and again resuspended in 567 ml of TE buffer. Then the suspension of 30 ml of 10% SDS and 3 ml of proteinase K (20 mg mL-1) was mixed uniformly and incubated at 37°C for one and half hours. After completion of the incubation the 100 ul of 5 mol L^{-1} NaCl and 80 ml of CTAB buffer containing 10 g CTAB and 4.1 g NaCl in 100 ml of water was added and again incubated at 65°C for 10 minutes in water bath. Finally after deproteination centrifugation was done at 10000 x g and after discarding the supernatant, the pallet was mixed with double volume of isopropanol (70%). Again centrifugation was done at 10000 x g for 10 minntes at 4°C. Finally the supernatant was discarded and the pellet was resuspended in 20µ TE buffer and stored at -20°C.

3.8.2: PCR technique:

Polymerase chain reactions (PCR) were performed using a MJ Mini Personal Thermal Cycler (Bio-Rad) with the help of gene specific primer. A set of 16S

rRNA primer as suggested by Boudazin *et al.* (1999) and a set of primer named Rsol_*fliC* suggested by Sconfeld et al. (2003) for identification of *Ralstonia solanacearum* were used. Twenty-five microliter reaction mixture was prepared and 2 µl of genomic DNA was taken as template. Amplicons were analyzed by electrophoresis in 1% agarose gel. Finally the amplicons were cloned.

- **List of primers used for PCR amplification**

Primer	Primer sequence	Reference
16S rRNA	Forword 5' – GAGTTTGATCATCATCGCTCAG – 3' Reverse 5' – GGCGGGACTTAACCAACAT – 3'	Boudazin *et al.* (1999)
Rsol_*fliC*	Forword 5' – GAACGCCAACGGTGCGAACT – 3' Reverse 5' – GGCGGCCTTCAGGGAGGTC – 3'	Sconfeld et al. (2003)

- **PCR reaction mixture for 16S rRNA**

Go-Taq-Green Mastermix (2X)	12.5 µl
Forward primer	0.5µl
Reverse primer	0.5µl
Sample DNA	2.0µl
Nuclease free water	9.5µl
Total volume	25 µl

- **PCR reaction mixture for *fli*C gene**

Taq buffer (5X)	2.5µl
$MgCl_2$ (25mM)	1.5µl
dNTP mix (10mM)	2.0µl
Forward primer	0.5µl
Reverse primer	0.5µl
Sample DNA	2.0µl

Taq DNA Polymerase (5u/µl)	1.0µl
Sterile water	15.0µl
Total volume	25 µl

3.8.3: Detection of PCR amplicons in agarose gel

The PCR amplicons of the bacterial 16s rRNA genes and *fli*C genes were resolved on 1% agarose gel containing ethidium bromide following methods described below (section 3.8.4). The resolved amplicons were observed under UV light in a UV transilluminator. The molecular weight of PCR products was measured by using molecular weight markers (100bp/500bp ladder, Promega) run parallel to samples.

3.8.4: Gel electrophoresis

Purified genomic DNA was subjected to agarose gel electrophoresis performed in a submarine gel electrophoresis system (Bangalore genei (India) Pvt. Ltd., India). Agarose was suspended in 1X TAE buffer (1%) and dissolved by heating in a water bath until clear solution was obtained. The solution was allowed to cool down to about 50-60^{o}C. Ethidium bromide (0.5µg/ml) was added mixed and poured into the gel casting tray. The solution was allowed to solidify and then it was completely submersed in electrophoresis tank containing 1X TAE running buffer. DNA sample (6µl) and gel loading buffer (2µl) was mixed and loaded onto wells. Electrophoresis was run at 60volt for 1 hour. The gel was then picked up from the tank and viewed under UV light in a UV transilluminator (Bangalore Genei (India) Pvt. Ltd, Bangalor, India). Bright fluorescent orange bands indicated the presence of DNA.

3.9: Cloning and sequencing of purified PCR product:

The pGEM-T vector (Promega, Madison, USA) was used for the cloning of purified nucleotide sequence (PCR products) following the method of Sambrook and Russel (2001).

3.9.1: Competent cell Preparation:

The *Escherichia coli* strain JM109 was used for the procedure. One cfu of fresh overnight grown culture of *Escherichia coli* strain JM109 was added to 10 ml Luria-Berteni broth (LB) and incubated at 37˚C for12-14 hours. Then 100 µl broth culture (overnight grown) was inoculated into same volume of freshly prepared LB broth and incubated for 2 to 4 hours at 37˚C. Then centrifugation has done at 6000 rpm for 7 min to harvest the bacterial cells in1.5 ml microcentrifuge tube. After discarding the supernatant, 750 µl of solution- I (solution of 80mM $MgCl_2$ and 20mM $CaCl_2$) was added and mixed gently. Again centrifuged at 3000 to 4000 rpm for 5 to 7 min and supernatant was discarded. Then 750 µl solution - II (100mM $CaCl_2$ solution) was added as same volume as solution - I and mixed gently. The mixture was incubated for 45 minutes at 4˚C. After incubation the mixture was taken for centrifuge at 3000 to 4000 rpm for 5 to 7 minutes and supernatant was discard. Then finally 500 µl of solution – II was added and stored at -70°C.

3.9.2: Transformation:

A suitable quantity (2 to 10 µl) of ligation mixture and 100 µl of competent cell suspension was mixed carefully and the mixture was incubated at 4°C for 30 to 60 minutes. Then the heat shock therapy was applied by incubating the solution at 42°C for 90 seconds and again at 4°C 10 minutes. 300 µl LB was added to the solution and incubated at 37˚C for 2 hours in shaker incubator (Remi, India). The centrifugation has done at 6000 rpm for 5 minutes for harvesting the transformed bacterial cells and supernatant was discarded. Finally the precipitate was resuspended with 100 µl of fresh LB broth and plated in 20 ml LB agar plate containing 4 µl 20% Isopropyl thiogalactoside, or isopropyl beta-D-thiogalactopyranoside (IPTG), 20 µl 4% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and 20 µl 50 mg/ml ampicillin.

- **Ligation mixture**

Ligation buffer:	5.0 µl
Ligation buffer:	5.0 µl
pGEM-T vector:	0.5 µl
T4 DNA ligase:	1.0 µl
Purified RT-PCR product:	1.5 µl
Sterile distilled water:	2.0 µl

- **Blue-white Screening**

Among the blue and white colonies in the ampicillin plate a single white colony was isolated and picked up through a sterile tooth pick and kept into a 1.5 ml micro centrifuge tube containing 200 µl of sterile distilled water. Then the mixture was taken to boil at 100°C for 10 minutes and cooled immediately, then centrifuged at 7000 rpm for 5 minutes at 4˚C. Finally the supernatant was transferred into a fresh 1.5 ml micro centrifuge tube. For PCR reaction 2 µl supernatant was used.

- **PCR reaction mixture for cloned DNA**

Taq buffer (5X)	5µl
$MgCl_2$ (25mM)	1.5µl
dNTP mix (10mM)	1µl
Forward primer	1 µl
Reverse primer	1 µl
Sample DNA	2.0µl
Taq DNA Polymerase (5u/µl)	0.125µl
Sterile water	13.375µl
Total volume	25 µl

3.9.3: Purification and sequencing of PCR products

GeneiPure™ Quick PCR purification kit was used for purification of the PCR products. Purification was done following the manufacturer's protocol. Binding

Buffer (5 volume) was added to PCR product (1 volume) and mixed well. GeneiPure™ column was placed into a 2 ml collection tube and the sample was loaded and centrifuged at 11,000 rpm for 1 minute. The flow through was discarded and the GeneiPure™ column was again placed in the collection tube and washed with of Wash Buffer I (500 µl) and centrifuged (at 11,000 rpm for 1 minute). Flow through was discarded and the GeneiPure™ column was again kept in to the collection tube. After that, one volume of Wash Buffer II was diluted with four volume of absolute ethanol (just before use). Again, the GeneiPure™ column was washed with 700 µl of diluted Wash Buffer II and centrifuged at 11,000 rpm for 1 minute. Flow through was discarded and the said column was placed back to the collection tube. It was then centrifuged at 11,000 rpm for 2 minutes to remove the traces of Wash Buffer and the collection tube was discarded. Thereafter the column was opened and placed in fresh and sterile 1.5 ml vial. It was then incubated at 70° C for 2 minutes for complete removal of ethanol. Finally, For elution of DNA, 50 µl of pre-warmed (in dry bath at 70° C for 5 minutes) Elution Buffer was added to the center of the column and incubated at room temperature for 1-2 minutes.

3.9.4: Sequencing of the cloned insert:

The cloned PCR products were sent to Xcelris Genomics Ltd. for sequencing. Annotation and BLAST analysis were done and deduced amino acid sequences were submitted to GenBank and necessary accession numbers were procured.

3.10: Phylogenetic analysis:

The 16s rRNA gene sequences and the *fli*C gene sequences of the pathogenic fungal strains were compared with other related gene sequences available in GenBank databases using the BLAST search facility at NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul et al. 1997). Thereafter the sequences were aligned with sequences of GenBank. The nucleotide identity matrices were formed with SDT 1.2 (Muhire et al. 2014). Neighbour-joining method of Saitou & Nei (1997) was used through Kimura-2 parameter in MEGA 6.0 (Tamura et

al. 2013) to generate phylogenetic trees following alignment with ClustalW 1.6 (Thompson et al. 1994). Confidence in the tree topology was determined by bootstrap analysis using 1000 re-samplings of the sequences (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2013).

3.11: Bacterial wilt disease management by indigenous isolated bacteria:

3.11.1: Screening and Isolation of antagonistic bacteria:

Procedure of isolation and screening of antagonistic bacteria from fields of the present study area have been presented in section 3.4.2.

3.11.2: Biochemical characterization of bacterial antagonists:

After Gram staining all such bacteria isolated were observed under light microscope to confirm their Gram positive nature. Finally the major biochemical tests were carried out for the identification of the isolates such as Catalase test, Citrate test, Indole test, Oxidase test and Voges Proskauer test as discussed in section 3.6.

3.11.3: Molecular characterization of bacterial antagonist:

The procedure of molecular characterization of bacterial antagonist by 16s rRNA study was same as discussed in case of bacterial pathogen R. solanacearum and has already been discussed in sections 3.8 3.9 & 3.10.

3.11.3: *In-vitro* antagonism study:

For the *in-vitro* study dual culture technique was followed. In nutrient agar plate one line of pathogenic bacteria was streaked by a sterilized needle, so that, the line is passed through the centre of the Petriplate. Thereafter one lines of antagonistic bacteria was streaked parallel to that line and left side and another line on the right side of the central line. Then the plates were

incubated at 30°C. Average breadth of the lines was observed and recorded for antagonism, if any, after 12 h, 24 h and 48 h.

3.11.4: *In-vivo* antagonism study:

3.11.4.1: Root disease assessment following antagonist application (By soil application)

At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. The pot was treated as inoculated control. In second set another 1.5 Kg sterilized soil was mixed with 100 ml of *Ralstonia solanacearum* (RSG01) suspension and also with 100ml of antagonist (*Bacillus cereus*; isolate HS01). Thereafter the soil was poured in a pot. Similarly third and fourth sets were prepared with broth of another two antagonistic isolates (HS02 and HS03). Fifth set was done where a mixture of three antagonistic isolates in equal volume was added. These sets were considered as inoculated-antagonist applied sets. All these soils were poured in separate pots and labeled. Sixth set was un-inoculated control where no application of antagonistic bacteria was done. Actually this set contained sterilized soil in a pot.

Healthy tomato plants of 25 days old were sown in the pots. For each treatment four plants were sown. Reduction of disease was calculated for each plant and finally average of three plants was recorded on the basis of wilting index. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.11.4.2 Challenge inoculation by pathogenic bacteria followed by Bacterization of roots of tomato plants and assessment of disease in field condition

Bacterization of the tomato roots of 25 days old plants (of cv.PKM1) was done by dipping in bacterial suspensions for two hours. After incubation, the plants were sown in soils in plots prepared in the experimental garden of the Department. After 24 hours, the plants were inoculated with pathogenic bacteria suspension. Bacterial suspensions were made from 48 hour old cultures in Nutrient broth. The 50 ml culture was centrifuged and the pellet of bacterium was suspended in 50 ml sterile distilled water. The resultant suspension was used for inoculation. About 50 ml suspension of pathogenic bacterium was applied at the base of each plant sown in the experimental plots except the plants of control plots. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.12: *In vitro* control of *Ralstonia solanacearum*

3.12.1: *In vitro* control of *Ralstonia solanacearum* with *Trichoderma harzianum* (following dual culture technique):

At the onset of the study one fungus *Trichoderma harzianum* (IRCC-4572) was cultured in potato dextrose agar (PDA) medium contained plate. Fresh sterilized PDA plates were taken for dual culture test. Mycelia block of 6 mm diameter were cut from a 5-day old culture plate of *T. harzianum* and was placed 2 cm away from the centre of the plate. One bacterium (*R. solanacearum* isolate RSG01) was streaked in straight line in the opposite side of the mycelia block and 2 mm apart from the centre of the same plate. Growth of bacterium and fungus were recorded.

3.12.2: *In vivo* evaluation of *Trichoderma harzianum* for control of disease*:*

In the present study one *Trichoderma harzianum* (IRCC-4572) was used against the wilt causing bacteria *Ralstonia solanacearum* isolate of tomato under pot condition following the method of Kumar and Ganesan (2006) with some modifications. At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. In a similar pot 100ml of *Trichoderma harzianum* spore suspension was mixed after inoculation with *Ralstonia solanacearum* (RSG01) broth. For this study 25 days old seedlings of cv. PKM-1 were taken. The fungal spore suspension was prepared from PDA plate grown at 25°C for 96 hours. The spores from the surface were suspended in sterile distilled water and were shaken well to make a homogenized spore suspension. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.13: Control of pathogenic bacteria by botanicals:

As chemicals are not much effective to control the bacterial wilt caused by *Ralstonia solanacearum*, some botanicals were used because of their natural origin and biodegradable nature. There are some reports of effective antibacterial activity.

- **Selection and collection of plants/plant parts for extraction:**

Eight plants were selected for extraction of botanicals. The selection was based on some previous reports and local availability. The plants used for the

purpose have been listed below along with their common name, family and parts used for extraction.

- **List of plants:**

Botanical name with family, parts used and common name	**Traditional uses**	**Anti bacterial use if any**
Spermacoce hispida L. (Family: Rubiaceae; Parts used: Twigs; Common name: Shaggy button weed)	Antifungal, anticancer and antioxidant properties. (Meti et al, 2013)	Extract used against bacteria. (Muthu et al. 2010)
Allium sativum L. (Family: Amaryllidaceae; Parts used: Bulbs; Common name: Garlic)	Stimulant, diuretic, expectorant, in flatulency and dysentery (Saha et al., 2005)	Extract used against bacteria. (Wolde et al., 2018)
Zingiber officinale Rosc. (Family: Zingiberaceae; Parts used: Rhizome; Common name: Ada)	Used in dyspepsia and flatulent colic (Saha et al., 2005)	Extract used against bacteria. (Malu et al., 2009)
Azadirachita indica A. Juss (Family: Meliaceae; Parts used: Leaves; Common name: Neem)	Applied to boils as poultice, decoction antiseptic, used in ulcers and eczema (Saha et al., 2005)	Extract used against *Ralsonia solanacearum* (Narasimha and Srinivas, 2012)
Ocimum sanctum L. (Family: Lamiaceae; Parts used: Leaves; Common name: Tulsi)	Fresh leaves bruised and applied externally for curing ring worm and other skin diseases	Antibacterial (Singh et al., 2005; Mishra and Mishra 2011)

	(Saha et al., 2005)	
Cinamomum tamala (Buch.-Ham.) T.Nees & C.H.Eberm (Family: Lauraceae; Parts used: Leaves; Common name: Tejpata)	Medicinal use in skin rashes, earaches, and rheumatism also used in cooking. (Batool et al.,2020)	antibacterial, antiviral, antifungal activity, insect repellant activity, (Batool et al.,2020)
Camellia sinensis (Family: Theaceae; Parts used: Leaves; Common name: Tea)	Traditionally used as beverage drink. Several health benefits are there in tea	Antibacterial (Kawamura and Takeo,1989; Vasudeo and Sonika, 2009)
Thuja occidentalis (Family: Cupressaceae; Parts used: Leaves; Common name: Eastern white ceder)	Traditionally used in bronchitis, Rheumatism (Tsiri et al., 2009)	Antibacterial (Sah et al., 2017)

- **Alcoholic extraction Protocol:**

Plant parts (100g) were taken and cut into small pieces. The sample was mixed with 50 ml of sterilized distilled water and macerated in a pestle and morter under aseptic condition. After maceration 50ml of absolute alcohol was added. The mixture was kept for one hour in a sealed glass tube. After one hour the mixture was squeezed through a sterilized muslin cloth so as to get a crude liquid extract. The crude extract was centrifuged at 1000 rpm for 5 minutes. The filtrate was concentrated so that alcohol is removed. Again 50 ml sterile distilled water was added to it and final volume was adjusted to 100 ml. Thus concentration of crude extract became 1g/ml.

- ***In-vitro* evaluation of plant extracts:**

Extracts of the selected plants were screened at different dilutions viz., Crude extract, 50%, 25% and 12.5% diluted. The efficacy of the extracts was tested by the zone of inhibition assay technique against *Ralstonia solanacearum* causing bacterial wilt of tomato. Bacterial suspension (5 x 10^8cfu/ml) was seeded to the sterilized nutrient agar medium by mixing the bacterial culture with the cooled nutrient agar (40 to 50°C) in a 500ml conical flask. The seeded medium was poured in sterilized Petri plates and was allowed to solidify. Five wells each of 5 mm diameter were dug in each plate. One well at the centre and four wells were dug at peripheral region keeping some distance from the margin. In the central well 50 µl of sterile distilled water was placed. In peripheral wells 50 µl plant extracts each of four different concentrations were placed. The plates were incubated first at 5±1°C for 5 hours to allow the diffusion of the extract into the medium. The plates were then incubated at 28±1°C for 72 hours. Diameter of zone of inhibition produced around the wells in each plant extract at different dilutions were measured and recorded. No inhibition zone was observed around central well kept as control.

- ***In-vitro* evaluation of plant extracts:**

In the present study one plant extract (leaf extract of Camellia sinensis) was used against the wilt causing bacteria *Ralstonia solanacearum* isolate of tomato under pot condition following the method of Kumar and Ganesan (2006) with some modifications. At first 1.5 Kg of garden soil was taken in plastic bag and sterilized in an autoclave. The autoclaved soil (1.5Kg) was mixed with 100ml of *Ralstonia solanacearum* (RSG01) broth. The mixture was semidried in room temperature and finally poured in a fresh pot. In a similar pot 100ml of *Camellia sinensis* leaf extract was mixed after inoculation with *Ralstonia solanacearum* (RSG01) broth. For this study 25 days old seedlings of cv. PKM-1 were taken. The fungal spore suspension was prepared from PDA plate grown at 25°C for 96 hours. The spores from the surface were suspended in sterile

distilled water and were shaken well to make a homogenized spore suspension. For each treatment 4 plants were sown. Finally the wilting index of the plants was recorded on the basis of visual observation. *Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

3.14: Induction of Disease resistance

3.14.1: Application of inducer-chemicals on tomato plants and inoculation

Roots of 15 days old fresh healthy tomato plants of (cv. PKM-1) were soaked in inducer chemicals [dissolved in sterile distilled water (10^{-3} M)] for 1 hour and finally sown in fresh earthen pots containing sterilized soil. Tree different chemicals *viz.* β-aminobutyric acid (BABA), Salicylic acid (SA) and Abscisic acid (ABA) were used as inducers to elicit resistance in the plants. The inducers were mixed with Tween-20 before spraying in order to ensure adhering.

One hour after treatment, treated plants were challenge inoculated with bacterial suspension of *R. solanacearum*. Experiments were performed in four sets (untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated). Plants were maintained in green house in a sterile environment under normal daylight condition.

The roots of untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated plants were harvested for studying expression of defense related enzymes after 0 day to 5 day at one day intervals following inoculation by the pathogen. Disease index was also recorded and computed and compared assessment of induction if any.

3.14.2: Extraction and estimation of defense related enzymes

Three different defense related enzymes were studied because of their potentiality to elicit defense against bacterial pathogens. The three different enzymes were Phenylalanine ammonia lyase, Peroxidase and polyphenol oxidase.

3.14.2.1: Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5):

- **Extraction:**

PAL activity was determined following the method of Sadasivam and Manickam (1996). PAL activity is the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290nm. One gram of root was harvested washed and dipped in liquid nitrogen for 10 minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 0.25M borate buffer (pH-8.7). The paste was filtered through muslin cloth. The filtrate was centrifuged [at 12500g for 15 minutes] at 4 °C. The supernatant was used as enzyme stock.

- **Enzyme assay:**

Borate buffer [0.25M] (0.5ml), Enzyme stock (0.25ml), Distilled water (1.5ml) and L-phenylalanine [0.1M] 1ml were mixed. The mixture was kept at 30°C for 30 minutes for reactions. Reaction was stopped by 0.5ml of 1M Trichloroacetic acid. Absorbance was recorded at 290nm in UV-VIS spectrophotometer. Enzyme activity was represented as μmol $min^{-1}g^{-1}$ fresh weight tissue using trans cinnamic acid as standard. PAL activity =

(Concentration x Dilution factor x Final volume x 3) / (Initial weight x Volume of enzyme x 60)

3.14.2.1: Peroxidase (POD) (EC 1.11.1.7):

 # Extraction:

POD activity was tested following the method of Hammerschmidt *et al.,* 1982. One gram of root was harvested washed and dipped in liquid nitrogen for 10

minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 0.01M sodium phosphate buffer (pH 6.5). The paste was filtered through muslin cloth. The filtrate was centrifuged [at 6000g for 15 minutes] at 4 °C. The supernatant was used as enzyme stock.

 # Enzyme assay:

Guaiacol [0.05M] (1.5ml) and Enzyme stock (200µl) were mixed in a cuvette which was placed in a UV-VIS spectrophotometer and initial reading was adjusted to zero at 420 nm. H_2O_2 (1% v/v) 100 µl was added in the cuvette and the changes in absorbance values was recorded up to 5 minutes at 1 minute intervals. Change in absorbance [$\Delta A_{420} min^{-1} g^{-1}$ fresh weight tissue] of 0.001 was considered as unit of enzyme activity. Peroxidase activity =

(Mean OD x Final volume x 10 x Dilution factor) / Initial weight

3.14.2.2: Poly-phenol oxidase (PPO) (EC 1.14.18.1):

 # Extraction

PPO assay was performed following the method of Sadasivam and Manickam (1996). One gram of root was harvested washed and dipped in liquid nitrogen for 10 minutes. Then the roots were taken in a pre-chilled morter and pestle and crushed with 5ml of 50mM Tris-HCl buffer (pH-7.2) containing 0.4M sorbitol and 1.0mM NaCl. The paste was filtered through muslin cloth. The filtrate was centrifuged [at 12000g for 10 minutes] at 4 °C. The supernatant was used as enzyme stock.

 # Enzyme assay:

Sodium phosphate buffer [0.1M, pH-6.5] (2.5ml) and Enzyme stock (0.2ml) were mixed in a cuvette which was placed in a UV-VIS spectrophotometer and initial reading was adjusted to zero at 495 nm. Catechol [0.01M] 0.3ml was added in the cuvette and the changes in absorbance values were recorded up to 5 minutes at 1 minute intervals. Enzyme activity was expressed as change in

absorbance. Enzyme activity = K*($\Delta A min^{-1}$) µmol $min^{-1} g^{-1}$ fresh weight tissue. PPO activity =

K x ΔO.D. x Final volume x dilution factor x 10 / initial weight x 1 minute.

[Where K=0.272 for PPO]

Chapter-4

RESULTS

4.1: Chapter-I: Bacterial wilt disease of tomato in fields of North Bengal and isolation of pathogen

4.1.1: Survey of bacterial wilt disease in North-Bengal

At the beginning of the present study, some districts of sub-Himalayan West Bengal (Coochbehar, Uttar Dinajpur and Dakshin Dinajpur) were surveyed for occurrence of bacterial wilt disease in tomato. These three districts are well known for cultivation of tomato. All together 26 different locations were found to be prone to bacterial wilt disease out of 50 different places/locations surveyed. The places were selected on the basis of wilt disease symptoms observed visually during survey. For further study and for collection of diseased samples those 26 locations were considered. Ten locations of Haldibari and three locations of Ghoksadanga both of Coochbehar district were found to show severe bacterial wilt disease symptoms in the cultivated tomatoes. Eight locations of Balurghat of Dakshin-Dinajpur district and four locations of Durgapur of Uttar-Dinajpur district were also found to show severe wilt symptoms during survey (Table 4.1 & Fig. 4.1). Diseased samples were brought to the laboratory and were subjected to isolation of bacterial pathogens.

4.1.2: Bacterial wilt disease symptom:

Infected plants start wilting. Gradually the plants become dry. No spots or yellowing was observed. In severe cases the whole plant topples and death of the plant occurs. When the main stem near the crown region were cut vertically, brown to black discoloration was evident inside the stems. When the infected plant stem was cut transversely at the base and was introduced into clean water, a milky white streaming strand was observed. The milky white stream is commonly called as bacterial ooze. This streaming of ooze continues for few minutes from severely infected plants. Streaming of bacterial ooze is a preliminary test for screening of bacterial wilt of tomato from that of fungal wilt (Fig.4.2).

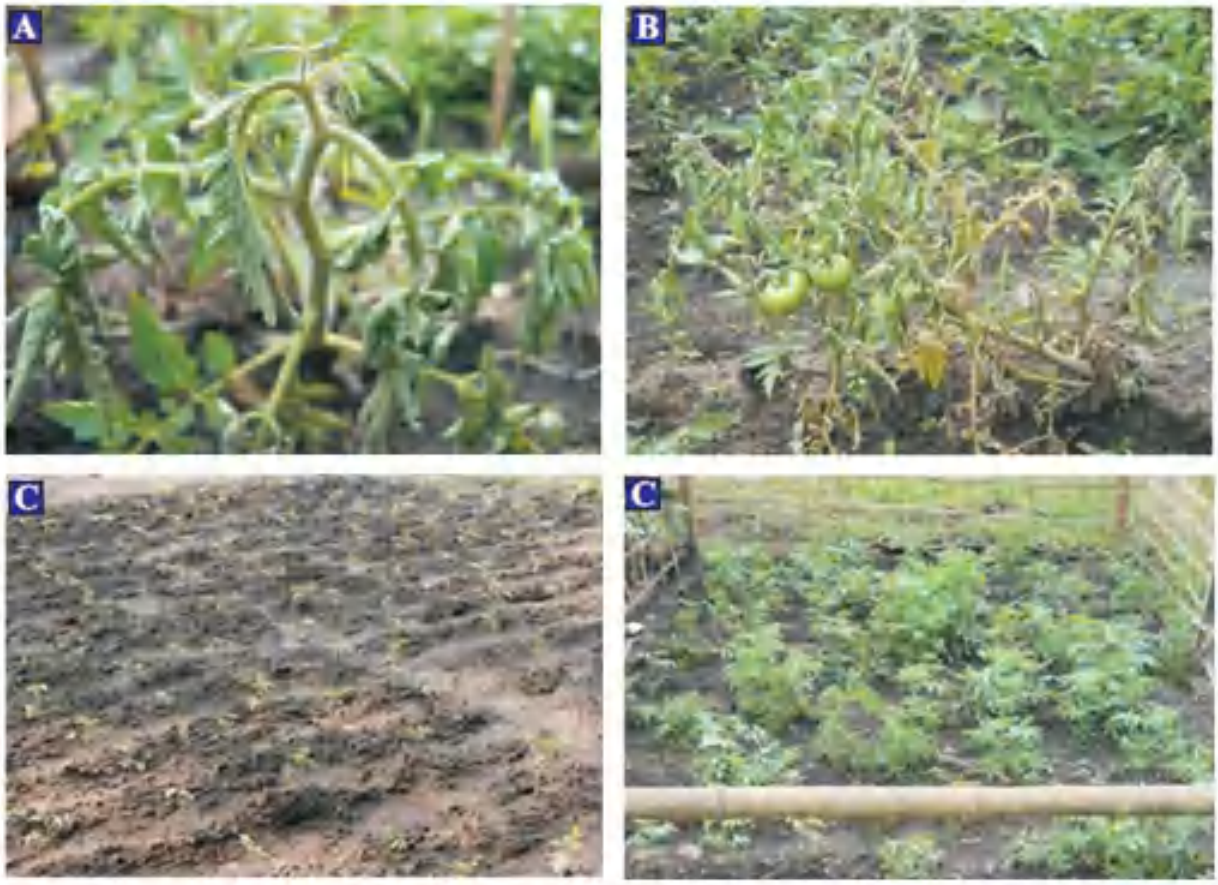


Fig 4.1: Infected plant in farmers field. a) Sonapur - Cooch Behar b) Haldibari - Cooch Behar c) Durgapur-Raiganj d) Ghokshadanga - Cooch Behar

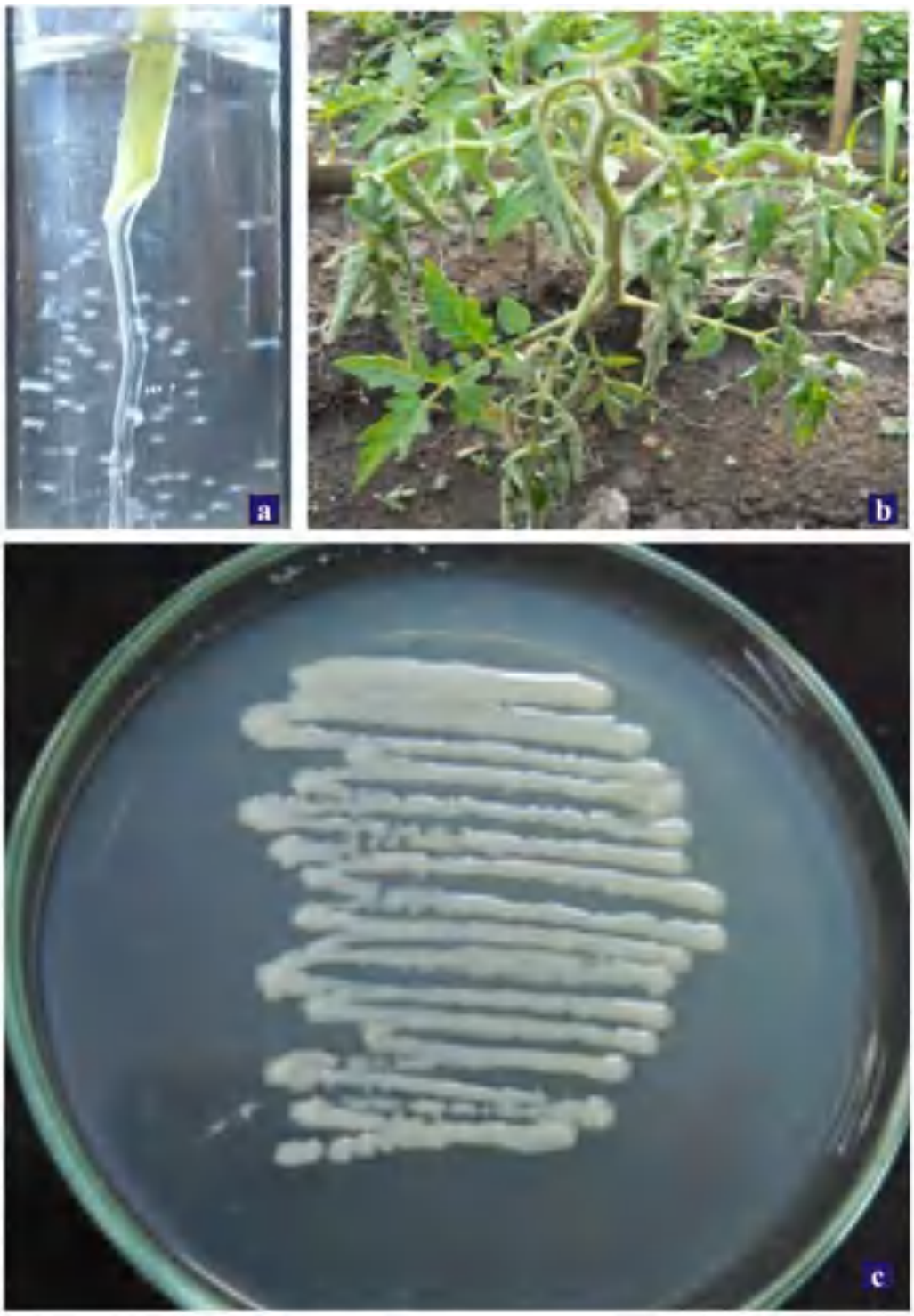


Fig. 4.2 : Isolation of bacteria from bacterial ooze. a) Collection of bacterial ooze in sterile distilled water b) Intected tomato plant from where ooze was collected . c) Pure culture isolation from bacterial Ooze.

4.1.3: Isolation of *Ralstonia solanacearum* from infected plants:

4.1.3.1: Isolation of bacteria from diseased plants:

In the present study all together 26 pathogenic bacteria were isolated from suspected bacterial wilt diseased tomato plants. All those plants showed bacterial ooze in sterilized distilled water were taken into consideration. The plants which did not show milky white bacterial ooze stream in clean water were discarded. Procedure of isolation has been discussed in materials and methods section 3.4.1. All such bacteria have been listed in Table 4.1. Photographic representation of some isolated bacterial plates has been shown in Fig. 4.3. Out of the 26 bacterial isolates ten isolates was collected from Haldibari. Haldibari is situated in the west Cooch Behar district and huge quantity of tomato is produced in Haldibari. Three isolates have been found from Ghoksadanga, situated in the central Cooch Behar district. Four isolates have been found from Durgapur region of Uttar Dinajpur District. Durgapur is also a place of substantial tomato production. Balurghat, of Dakshin Dinajpur district is another place where tomato is grown in large quantities. Nine bacterial isolates were also found from the Balurghat. All the isolated bacteria were purified by single colony isolation. Just after isolation each isolate was assigned isolate code. Three most virulent isolates (on the basis of pathogenicity test) were selected for molecular studies. Those three isolates were reassigned codes as RSG01, RSG02 and RSG03 before submission of gene sequences to GenBank.

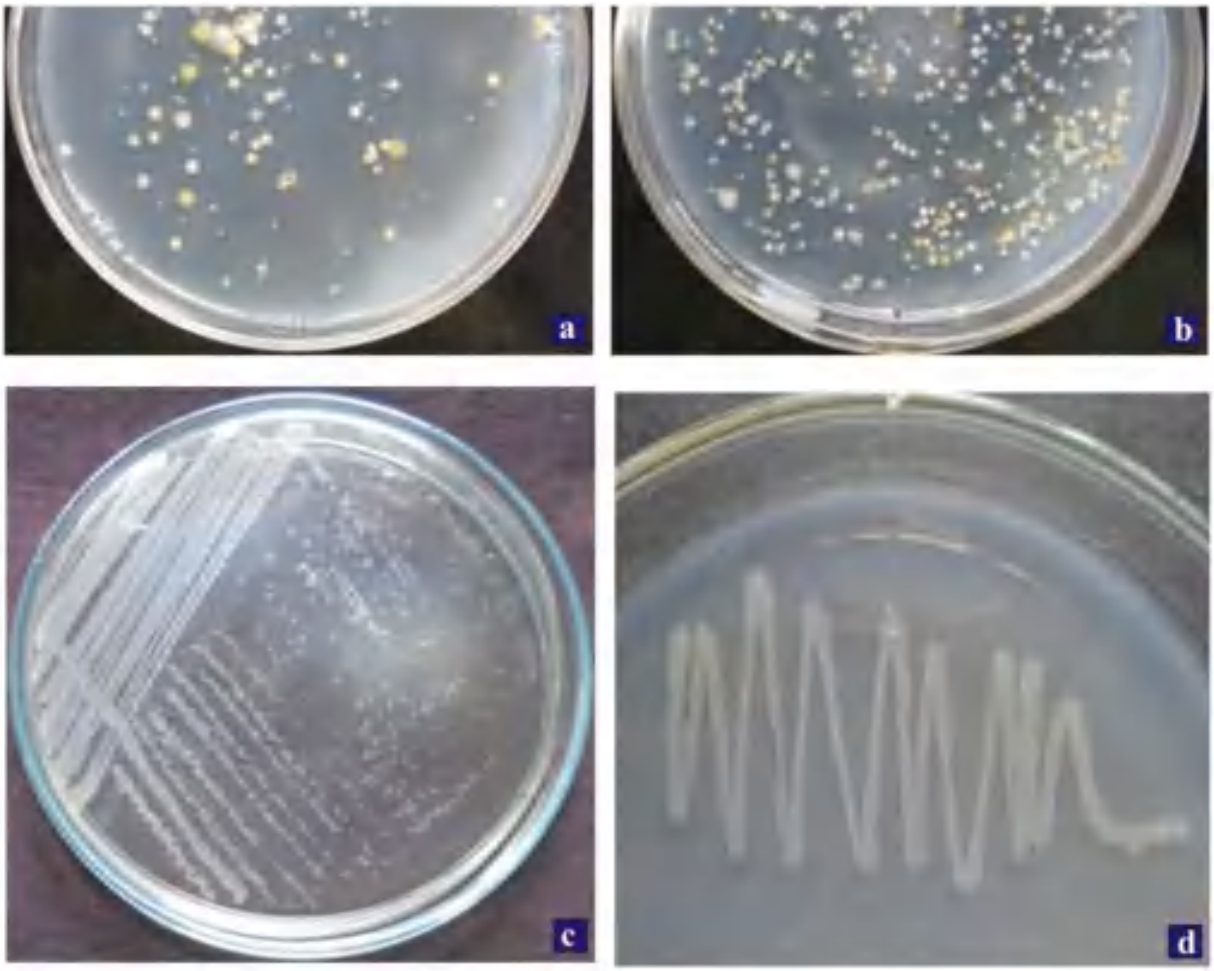


Fig. 4.3 : Isolation of bacteria by serial dilution and streaking. a) 10-4 Concentration plate b) 10-3 Concentration plate c) Single colony streak d) Pure culture isolation.

Table: 4.1: Isolation of bacteria from infected plants of the places studied.

Sl. No.	Place of collection of diseased samples	Initial code of isolate	Final code assigned during submission of gene sequence in Genbank
1	Haldibari (Cooch Behar)*	T1	--
2	Haldibari (Cooch Behar)	T4	--
3	Haldibari (Cooch Behar)	T5	--
4	Haldibari (Cooch Behar)	T6	RSG01
5	Haldibari (Cooch Behar)	D1	--
6	Haldibari (Cooch Behar)	D2	--
7	Haldibari (Cooch Behar)	D3	RSG02
8	Haldibari (Cooch Behar)	D4	RSG03
9	Haldibari (Cooch Behar)	D5	--
10	Haldibari (Cooch Behar)	D6	--
11	Durgapur (Uttar Dinajpur)	A1	--
12	Durgapur (Uttar Dinajpur)	A2	--
13	Durgapur (Uttar Dinajpur)	A3	--
14	Durgapur (Uttar Dinajpur)	A4	--
15	Balurghat (Dakshin Dinajpur)	G1	--
16	Balurghat (Dakshin Dinajpur)	H1	--
17	Balurghat (Dakshin Dinajpur)	H4	--
18	Balurghat (Dakshin Dinajpur)	H5	--
19	Balurghat (Dakshin Dinajpur)	H6	--
20	Balurghat (Dakshin Dinajpur)	H7	--
21	Balurghat (Dakshin Dinajpur)	H8	--
22	Balurghat (Dakshin Dinajpur)	H9	--
23	Balurghat (Dakshin Dinajpur)	H10	--
24	Ghoksadanga (Cooch Behar)	S1	--
25	Ghoksadanga (Cooch Behar)	S2	--
26	Ghoksadanga (Cooch Behar)	S3	--

*Names given in parentheses are the name of the districts of the collection spot. **--** = Not considered for sequencing and molecular studies.

4.2: Chapter II: Pathogenicity of isolated bacterial isolates

Pathogenicity of 26 bacterial isolates was tested on healthy tomato plants of a widely cultivated local variety. The plants were maintained in pots in the experimental garden. Details of the methods of inoculation, incubation conditions and assessment of disease have been discussed in materials and methods section 3.5.1. From the pathogenicity test results 3 bacteria were found to be highly pathogenic to moderately high pathogenic. 13 bacteria was pathogenic and 10 bacteria were weakly pathogenic (Table 4.2)

Table: 4.2: Pathogenicity of isolated *Ralstonia solanacearum* isolates in locally grown potted tomato plants.

Sl. No.	Initial code of isolates	Wilting Index (in the experimental pot condition) Days after inoculation					Pathogenicity status	Re-isolation of bacteria and confirmation of Koch's postulation
		3	6	9	12	15		
1	T1	-	-	+	+	++	Pathogenic	Yes
2	T4	-	-	-	+	++	Pathogenic	Yes
3	T5	-	-	-	-	+	Weakly pathogenic	Yes
4	T6/RSG 01	-	++	++	+++	+++++	Highly pathogenic	Yes
5	D1	-	-	-	+	++	Pathogenic	Yes
6	D2	-	-	-	-	+	Weakly pathogenic	Yes
7	D3/RSG 02	-	+	++	++	+++	Moderately high pathogenic	Yes
8	D4/RSG 03	-	+	++	++	+++	Moderately high pathogenic	Yes
9	D5	-	-	+	+	++	Pathogenic	Yes
10	D6	-	-	-	-	+	Weakly pathogenic	Yes
11	A1	-	-	-	-	+	Weakly pathogenic	Yes
12	A2	-	-	+	+	++	Pathogenic	Yes
13	A3	-	-	-	+	++	Pathogenic	Yes
14	A4	-	-	-	-	+	Weakly pathogenic	Yes
15	G1	-				++	Pathogenic	Yes
16	H1	-	-	-	-	+	Weakly pathogenic	Yes
17	H4	-	-	+	+	++	Pathogenic	Yes
18	H5	-	-	+	+	++	Pathogenic	Yes
19	H6	-	-	-	-	+	Weakly pathogenic	Yes
20	H7	-	-	-	-	+	Weakly pathogenic	Yes
21	H8	-	-	+	+	++	Pathogenic	Yes
22	H9	-	-	-	+	++	pathogenic	Yes
23	H10	-	-	+	+	++	pathogenic	Yes
24	S1	-	-	-	-	+	Weakly pathogenic	Yes
25	S2	-	-	-	-	+	Weakly pathogenic	Yes
26	S3	-	-	-	+	++	Pathogenic	Yes

Wilting index was graded as follows: + = up to 20% wilted; ++ = 20-40% wilted; +++ = 40-60% wilted and++++ = 60-80% wilted and +++++= 80-100% wilted.

4.2.1: Confirmation of Koch's postulations:

All the plants infected were subjected to re-isolation of bacteria. The reisolated bacteria were identified morphologically and biochemically. The identifications revealed that the isolated bacteria were same with the respective bacteria by which the plant was inoculated. Thus, Koch's postulations for all the 26 bacterial isolates were confirmed.

4.2.2: Screening of susceptible and resistant tomato plants:

To screen susceptible tomato plant varieties from the certified and cultivated tomato plant varieties (largely grown by the farmers of the present study area), were subjected to pathogenicity test by the most virulent *Ralstonia solanacearum* (RSG01). In this case, four varieties such as PKM-1, Vaishali, Rupali and Rashmi were taken in to consideration. 10 plants were taken per treatment (Table-4.3). In this case sterilized garden soil was used in the pots. Details of the procedure, inoculation technique and disease assessment has been discussed in materials and methods section 3.5.1.

From the results it was found that variety 'PKM-1' was most susceptible and variety 'Rashmi' was least susceptible against *Ralstonia solanacearum* (isolate RSG01) among the tested varieties. Varieties 'Vashali' and 'Rupali' were also susceptible but less susceptible than 'PKM-1' variety.

Table 4.3: Pathogenicity of most virulent *Ralstonia solanacearum* isolate RSG01 in four different certified varieties of tomato in Sterilized potted soil .

Plant variety of (*Lycopersicon esculentum*)	**Inoculated/control**	**Wilting index* (days after inoculation)**			
		3d	6d	9d	12d
PKM-1	Inoculated	-	++	+++	+++++
	Control (SDW)	-	-	-	-
Vaishali	Inoculated	-	+	++	++++
	Control (SDW)	-	-	-	-
Rupali	Inoculated	-	+	++	++++
	Control (SDW)	-	-	-	-
Rashmi	Inoculated	-	+	+	+++
	Control (SDW)	-	-	-	-
Wilting index was graded as follows: + = up to 20% wilted; ++ = 20-40% wilted; +++ = 40-60% wilted and++++ = 60-80% wilted and +++++= 80-100% wilted.-= no wilting.					

On the basis of pathogenicity test conducted on potted plants of local variety and screening of susceptible certified variety, it was considered to conduct pathogenicity test of 16 isolates (3 highly pathogenic and 13 less pathogenic bacteria) in field grown most susceptible certified tomato plants of variety 'PKM-1'. Details of the procedure of inoculation and disease assessment have been described in materials and methods section 3.5.2 & 3.5.1. Results of the said experiment have been presented in the table 4.4.

From the results it was found that almost all the tested isolates could induce wilting in the tested PKM-1 variety in field condition. It was also reconfirmed that three isolates were highly virulent on certified variety 'PKM-1'. The isolates RSG01, RSG02 and RSG03 could infect within 3 days of inoculation. After 15 days the plants inoculated with isolate RSG01 fully wilted and collapsed.

Table 4.4: Pathogenicity test of 16 virulent and moderately virulent isolates in field condition on susceptible tomato variety 'PKM-1'

Selected isolates	Wilting Index (Days after inoculation)				
	3	6	9	12	15
T1	-	+	++	++	+++
T4	-	+	++	+++	+++
T6 / RSG01	+	+	++	+++++	+++++
D1	-	+	++	++	+++
D3/ RSG02	-	+	++	+++	+++
D4/RSG03	-	+	++	+++	++++
D5	-	+	++	++	++++
A2	+	+	++	++	+++
A3		+	++	++	+++
G1	-	+	++	++	+++
H4	+	+	++	++	+++
H5	-	+	++	++	+++
H8	-	+	++	++	+++
H9	-	+	++	++	+++
H10	-	+	++	++	+++
S3	-	+	++	++	+++
Control (SDW)					

Percent disease symptom of wilt was recorded up to 15th day at 3 days intervals. Wilting index was determined by visual observation. '-' = No visual disease; + = 0-20% wilt observed; ++ = 20-40% wilting; +++ = 40-60% wilting; ++++ = 60-80% wilting; +++++ = 80 to 100% wilting. SDW= Sterile distilled water

 # Host range study:

As tomato plant belong to family solanaceae, hence it was considered to check infectivity of the most virulent isolate of tomato to three different solanaceous plants such as potato (*Solanum tuberosum* Variety: Kufri Jyoti), Brinjal (*Solanum melongena* variety: Muktakeshi) and Chilli (*Capsicum frutescens* variety: Kull Lanka) were used. Details of the procedure of inoculation and disease assessment were similar as followed in case of tomato. The procedure of experiment may be seen from materials and methods section 3.5.2 & 3.5.1. Results of the said experiment have been presented in the table 4.5.

From the results it was found that after 9 days of inoculation potato plants shown first wilting symptom and after 15 days of inoculation about 30 to 35 % disease symptom of wilting was evident. Brinjal and Chilli plants did not show any disease symptoms even after 15 days of inoculation by *Ralstonia solanacearum* (isolate T6/ RSG01)

Table 4.5: Host range study of virulent *Ralstonia solanacearum* (isolate T6/ RSG01) in three different plants of solanaceae

Plant name with variety inoculated	Wilting index* (days after inoculation)				
	3d	6d	9d	12	15
Solanum melongena (Variety: Muktakeshi)	-	-	-	-	-
Control (SDW)	-	-	-	-	-
Solanum tuberosum (Variety: Kufri Jyoti)	-	-	+	+	++
Control (SDW)	-	-	-	-	-
Capsicum frutescens (Variety: Kull Lanka)	-	-	-	-	-
Control (SDW)	-	-	-	-	-

Percent disease symptom of wilt was recorded up to 15th day at 3 days intervals. Wilting index was determined by visual observation. '-' = No visual disease; + = 0-20% wilt observed; ++ = 20-40% wilting; +++ = 40-60% wilting; ++++ = 60-80% wilting; +++++ = 80 to 100% wilting. SDW= Sterile distilled water

4.3: Chapter- III: Characterization of isolated pathogens

4.3.1: Morphological characterization

Twenty six pathogenic isolates of the present study was grown in Nutrient agar (NA) media and morphology of the pathogens along with colony characteristics, nature of broth when grown in Nutrient broth (NB), shape, occurance, size and sporulation if any were studied. The details of procedure of study have been described in materials and methods (section 3.7). Shape, size, sporulation and occurrence of bacteria along with sporulation (if any) were studied in light microscope. Colony characteristics were studied in on NA plates. The nature of growth of bacteria was studied in NB. The results have been presented in table 4.6. From the results presented in table 4.6 it was evident that no bacteria produced spores. In light microscopic study it was found that all the bacteria were rod shaped and occurred in single or in pairs. Colony morphology was mostly smooth, white and fluidal. In nutrient broth all the cultures were turbid with pellicle and sediments.

Casamino acid Peptone Glucose Agar (CPG) medium is generally used for growth of *Ralstonia solanacearum*. Hence, colony morphology of the three most virulent isolates was also studied on CPG medium (Table 4.7). In CPG medium shape of colonies of all the three bacteria were irregular and round. Colour of the colonies became reddish to deep red but surface was smooth and milky for all three bacterial colonies (Fig. 4.4).

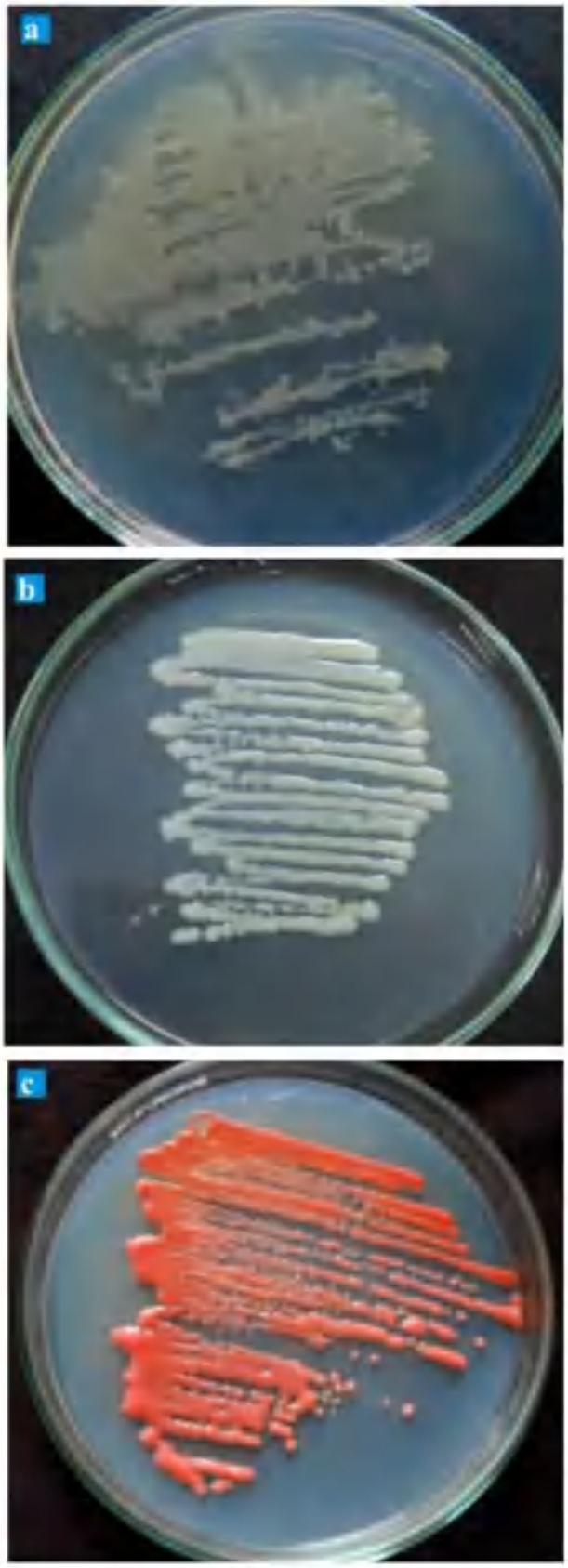


Fig.4.4: Growth of 3 different Ralstonia solanacearum (RSG01, RSG02 and RSG03) in CPG medium a) RSG01 b) RSG02 c) RSG03

Table 4.6: Morphological characters of the isolated bacterial pathogens

Bacteria Isolate	**Shape**	**Occurence**	**Size** (µm) length X Breadth	**Spore**	**Agar colonies**	**Culture in NB**
S1	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
S2	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
S3	rod	Single, pairs	2.7-3.1 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
D1	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
D2	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
D3/ RSG02	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
D4/ RSG03	rod	Single, pairs	2.8-3.0 X 0.9-1.3	-	Smooth, white fluidal	Turbid with pellicle and sediments
D5	rod	Single, pairs	2.8-3.0 X 0.8- 1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
D6	rod	Single, pairs	2.8-3.0 X 0.8- 1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
G1	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H1	rod	Single, pairs	2.8-2.9 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H4	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments

Bacteria Isolate	**Shape**	**Occurence**	**Size** (µm) length X Breadth	**Spore**	**Agar colonies**	**Culture in NB**
H4/RSG03	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H6	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H7	rod	Single, pairs	2.7-3.1 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H8	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H9	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
H10	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
T1	rod	Single, pairs	2.8-3.0 X 0.9-1.3	-	Smooth, white fluidal	Turbid with pellicle and sediments
T4	rod	Single, pairs	2.8-3.0 X 0.8- 1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
T5	rod	Single, pairs	2.8-3.0 X 0.8- 1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
T6 / RSG01	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
A1	rod	Single, pairs	2.8-2.9 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
A2	rod	Single, pairs	2.8-3.0 X 0.8- 1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
A3	rod	Single, pairs	2.5-3.0 X 0.8-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments

A4	rod	Single, pairs	2.7-3.1 X 0.7-1.0	-	Smooth, white fluidal	Turbid with pellicle and sediments
NB= Nutrient Broth						

Table 4.7: Colony morphology on CPG medium

Isolate Code	Shape of colony	Colour on CPG	Surface area
T6/ RSG 01	Irregular round shape	Slightly Reddish	Smooth and Milky
D3/ RSG 02	Irregular round shape	Reddish white	Smooth and Milky
D4/RSG 03	Irregular round shape	Reddish	Smooth and Milky
CPG medium = Casamino acid Peptone Glucose medium			

 # Motility Test of the isolated Bacteria

To check the motility of the isolated pathogenic bacteria all the bacteria were tested in motility medium. The procedure of the test has been described in materials and methods section 3.7.2. All the tested bacteria were found to be motile.

 # Scanning Electron Microscopy:

Three most virulent pathogenic isolates (RSG 01, RSG 02 and RSG 03) were subjected to electron microscopic study (Fig. 4.5). The details of the procedure of scanning electron microscopy have been discussed in materials and methods section 3.7.4.

From the figures the surface topography of the three bacteria were observed. From the photograph it was evident that the surface was more or less smooth but there were some depressions on the surface. In addition exact size of the tested bacteria was recorded. The size and shape of the three bacteria determined by the study has been presented in the table 4.8.

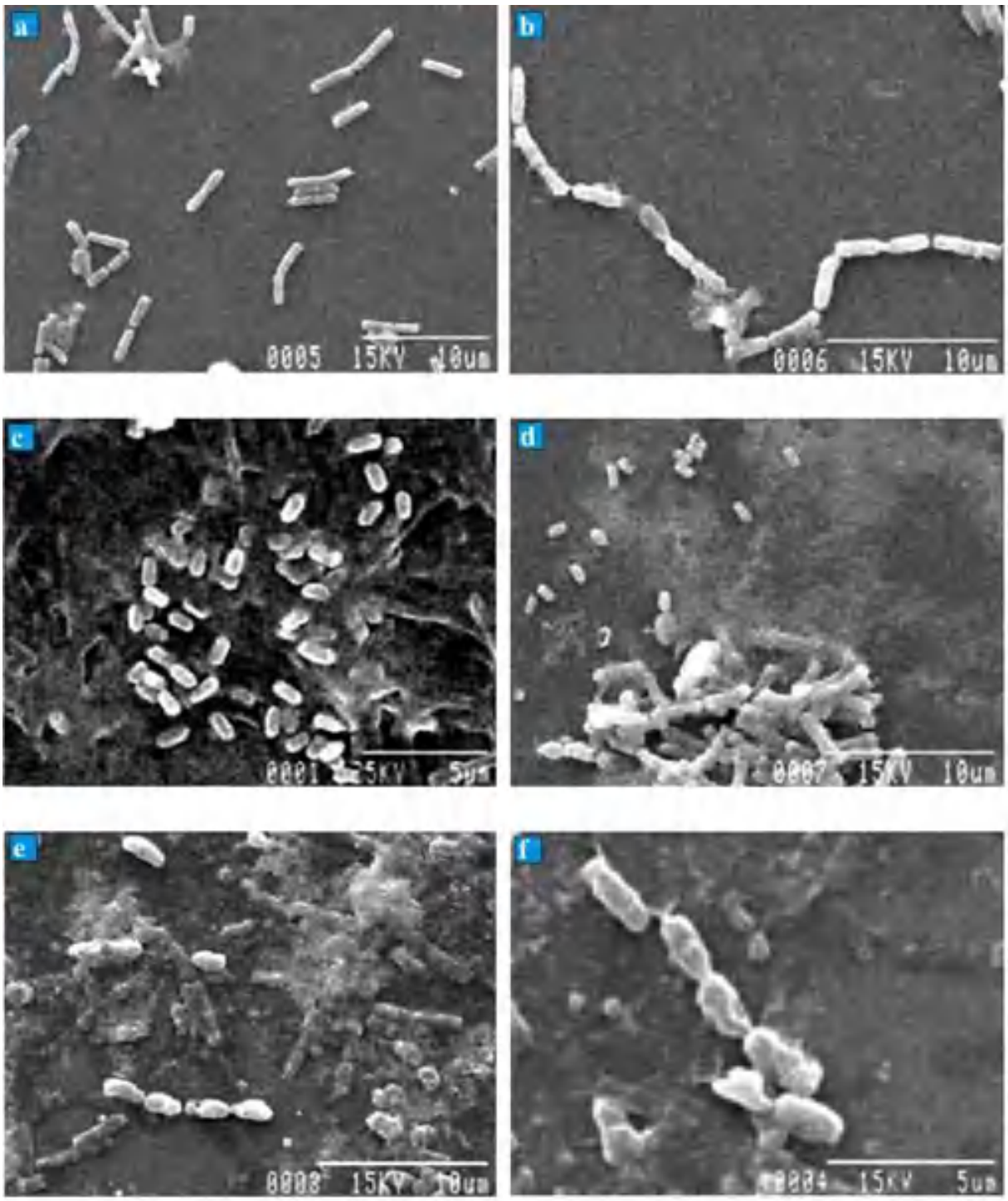


Fig. 4.5: Scanning electron micro photographs of 3 virulent bacteria RSG01, RSG02 and RSG03. a) & b) RSG01 isolate of (*Ralstonia solanacearum*) c) & d) RSG02 isolate of (*Ralstonia solanacearum*), e) & f) RSG03 isolate of (*Ralstonia solanacearum*),

Table 4.8: Shape and size of bacteria based on Electron Microscopy

Isolate Code	Shape	Size (Length/breadth in µm)
T6/RSG01	Rod shaped	3.028 x 0.93
D3/RSG02	Rod shaped	3.051 x 0.96
D4/RSG03	Rod shaped	3.081 x 1.335

4.3.2: Biochemical characterization of isolated pathogens.

The classical approach to identify bacteria is preliminary microscopic observation followed by Gram reaction which divides bacteria in two groups (Gram +ve and Gram –ve). After the broad classification of Gram reaction all the 26 isolated pathogens were subjected to biochemical tests to confirm their identification up to genus level as suggested by Trigiano et al. (2004). In the present study major biochemical studies conducted were Gram staining, aerobic/anaerobic growth condition study in Nutrient Agar and Nutrient Broth, Yellow pigmentation study on YDC medium, Growth on D1M medium, Growth below 4°C and above 40 °C, Oxidase test, Catalase test. Results of the above mentioned tests have been presented in the table 4.9.

From the results of Gram staining it was found that all the tested bacteria were Gram – ve (Fig. 4.6). Similarly all the bacteria tested for anaerobic growth could not grow indicating the aerobic nature of all the tested bacteria (Fig. 4.7). No bacteria could produce yellow pigmentation on YDC medium (Fig. 4.8). When NA plates of all the isolated bacteria were kept above 40°C, only four bacteria (isolates D4, H5, H10 and A3) could grow among the 26 bacterial isolates tested. Similarly when such plates were kept below 4°C, no bacterium could grow. In D1M media 14 bacteria could grow but others could not grow (table 4.9 & Fig.4.9). All the tested bacteria were oxidase test positive (Table 4.9 & Fig.4.10). Eight bacteria were catalase test positive (Table 4.9). When the isolates were

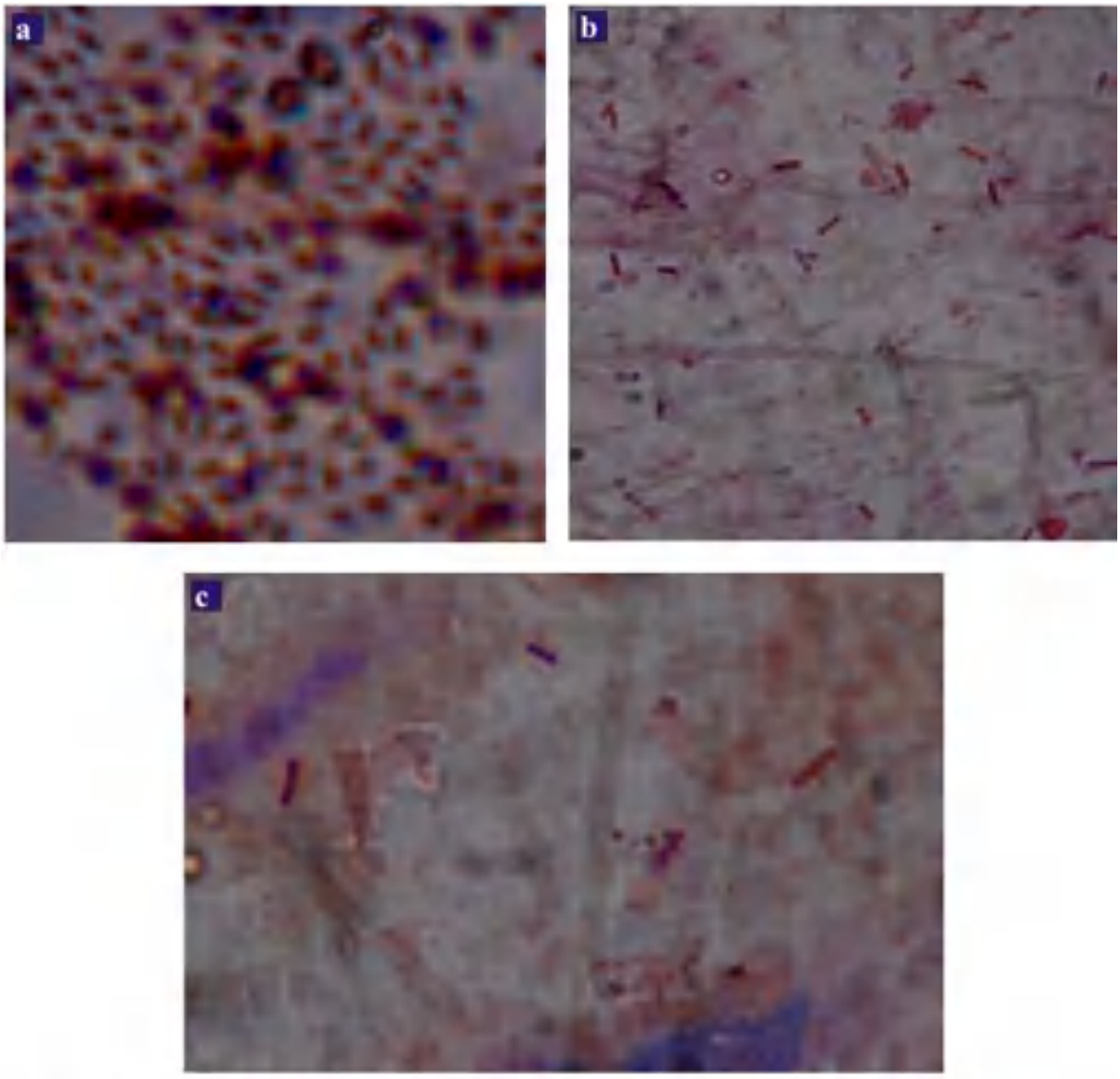


Fig 4.6: Morphological appearance of 3 verulent bacteria (*Ralstonia solanacearum*) a) Gram stained bacteria RSG01 b) Gram stained bacteria RSG02 c) Gram stained bacterial RSG03

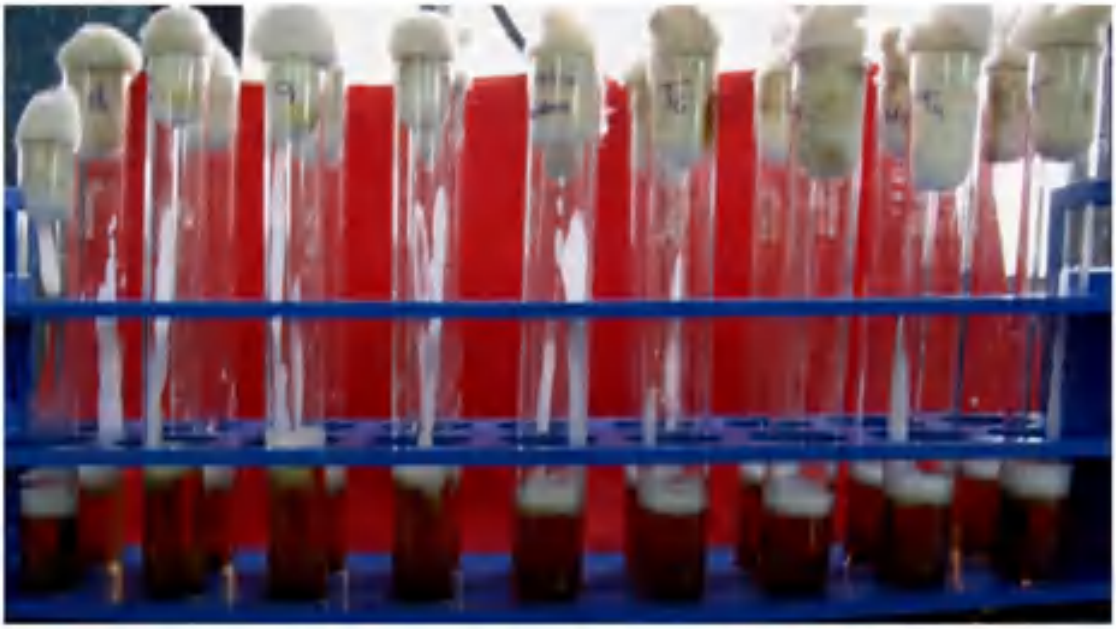


Fig 4.7: Anaerobic growth pattern test of the isolated cultures of Ralstonia solanacearum.

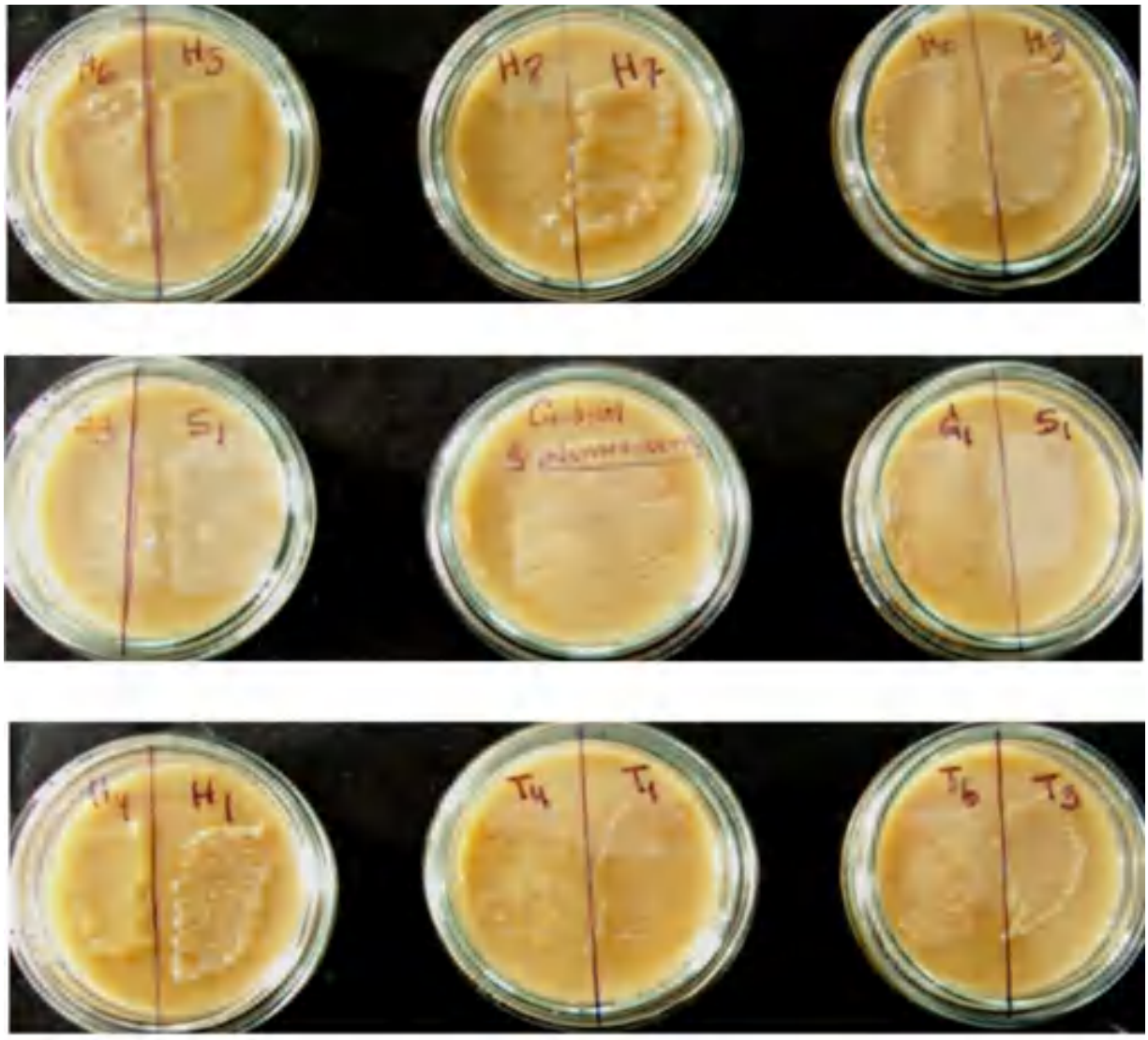


Fig. 4.8: Growth of 16 different cultures on YDC medium

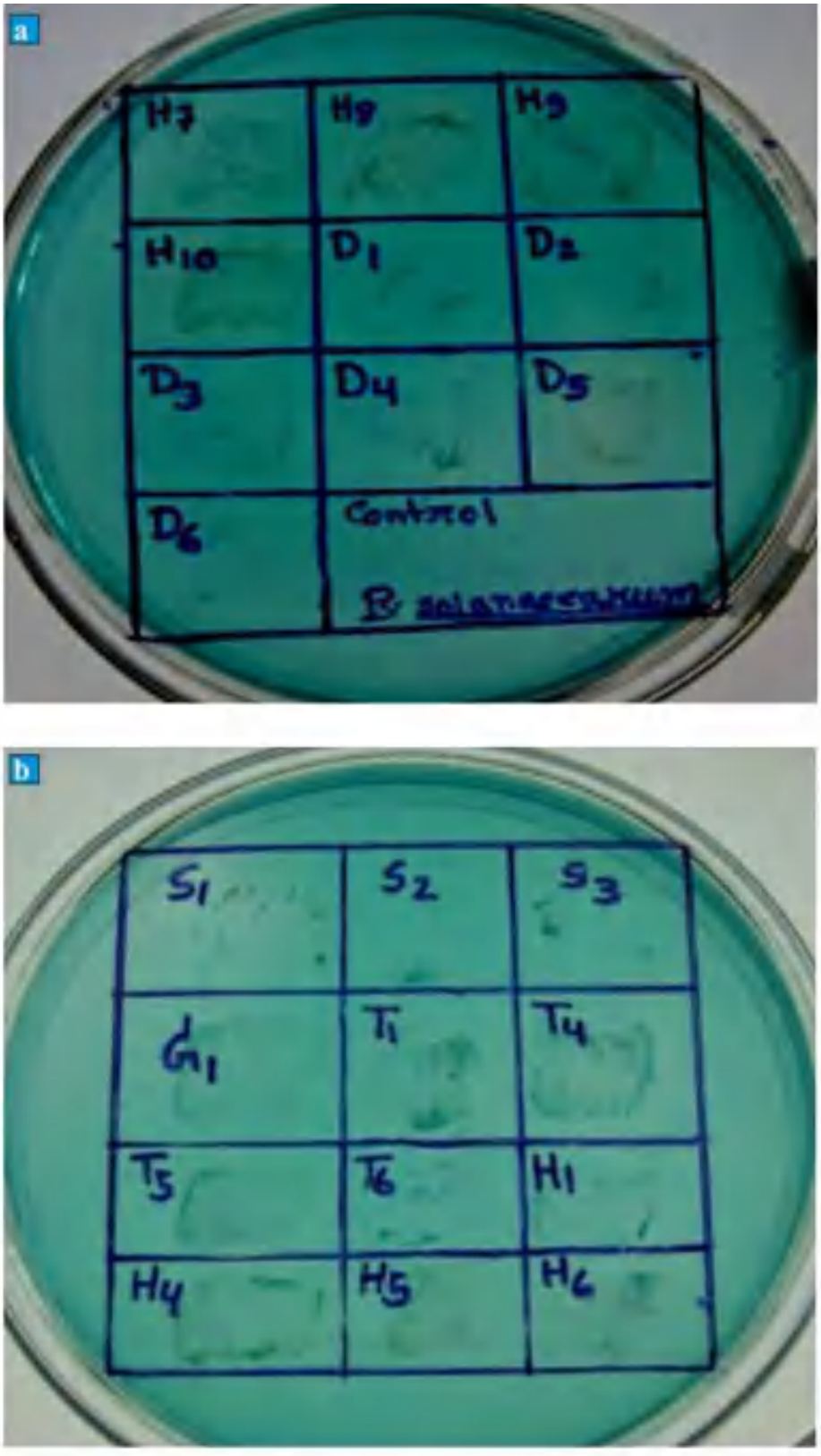


Fig. 4.9: a) & b) Growth of different Ralstonia solanacearum in D1M medium.

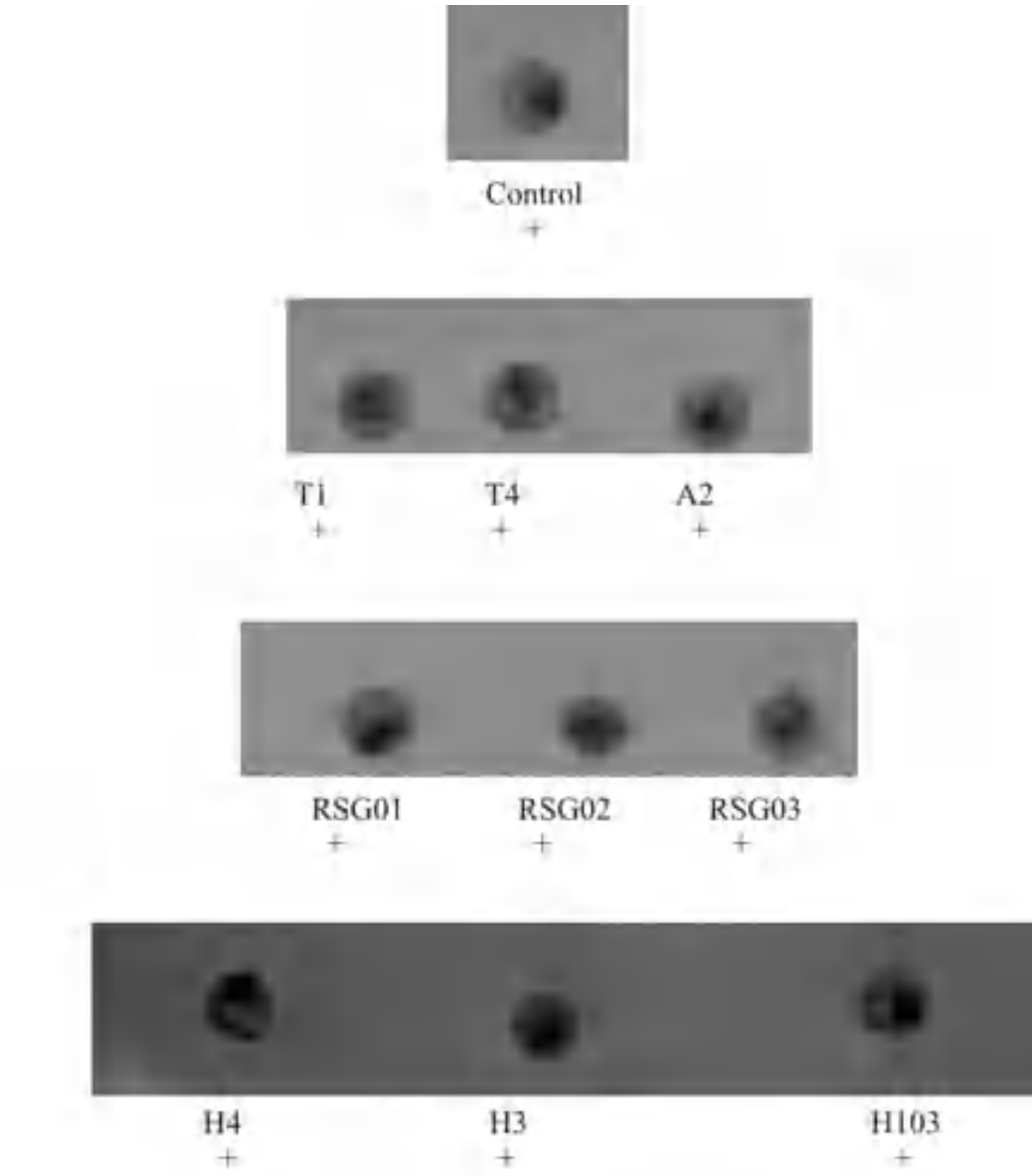


Fig 4.10: Oxidase test of some isolated bacteria

grown in PSA medium, only seven bacterial isolates could grow but others could not (Table 4.9 & Fig. 4.11).

Table 4.9: Biochemical characterization of the 26 *Ralstonia solsanacearum* isolates

Isolate code	Biochemical tests performed								
	Growth on PSA	Gram stain	Anaerobic growth	Yellow pigmentation on YDC medium	Growth above 40˚c	Growth below 4˚c	Growth on D1M medium	Oxidase test	Catalase test
S1	+	-	-	-	-	-	-	+	-
S2	+	-	-	-	-	-	-	+	-
S3	+	-	-	+	-	-	-	+	-
D1	+	-	-	-	-	-	-	+	+
D2	+	-	-	-	-	-	-	+	+
D3	-	-	-	-	-	-	-	+	+
D4	-	-	-	-	+	-	+	+	+
D5	+	-	-	+	-	-	+	+	+
D6	+	-	-	-	-	-	-	+	+
G1	-	-	-	-	-	-	-	+	-
H1	-	-	-	-	-	-	+	+	-
H4	-	-	-	-	-	-	+	+	-
H5	-	-	-	-	+	-	+	+	-
H6	-	-	-	-	-	-	+	+	-
H7	-	-	-	-	-	-	+	+	-
H8	-	-	-	-	-	-	+	+	-
H9	-	-	-	-	-	-	+	+	+
H10	-	-	-	-	+	-	+	+	+
T1	-	-	-	-	-	-	+	+	-
T4	-	-	-	-	-	-	+	+	-
T5	-	-	-	-	-	-	+	+	-
T6	-	-	-	-	-	-	+	+	-
A1	-	-	-	-	-	-	-	+	-
A2	-	-	-	+	-	-	-	+	-
A3	-	-	-	+	+	-	-	+	-
A4	-	-	-	+	-	-	-	+	-

After the biochemical tests conducted for the 26 isolates it was also considered to conduct different carbohydrate utilization tests with KB009 HiCarbohydrate™ Kit. This experiment were performed on three virulent isolates (RSG01,RSG02 and RSG03) only. The kit included Indole test, Methyl

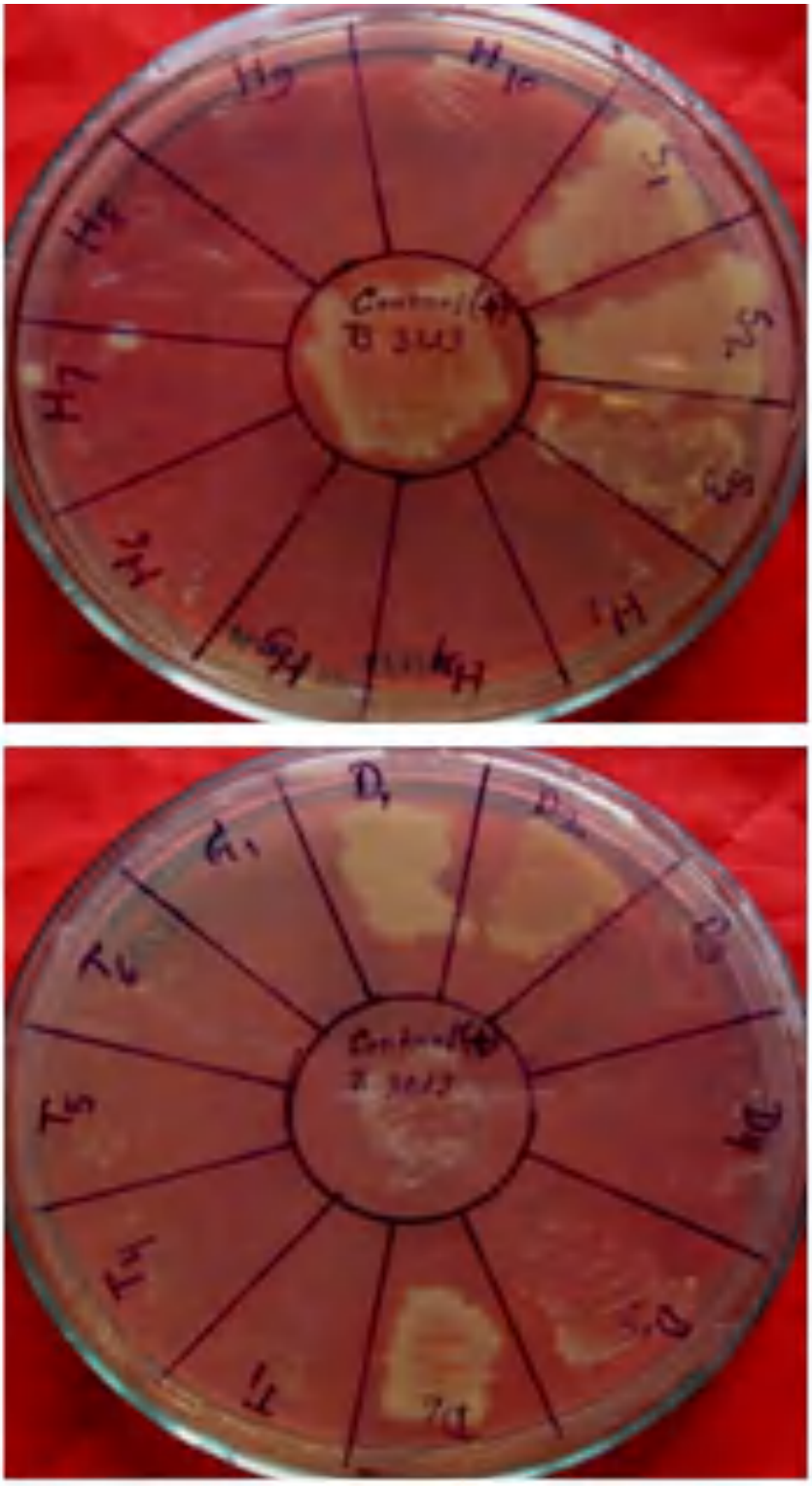


Fig. 4.11: Growth of different isolated and purified cultures on PSA medium.

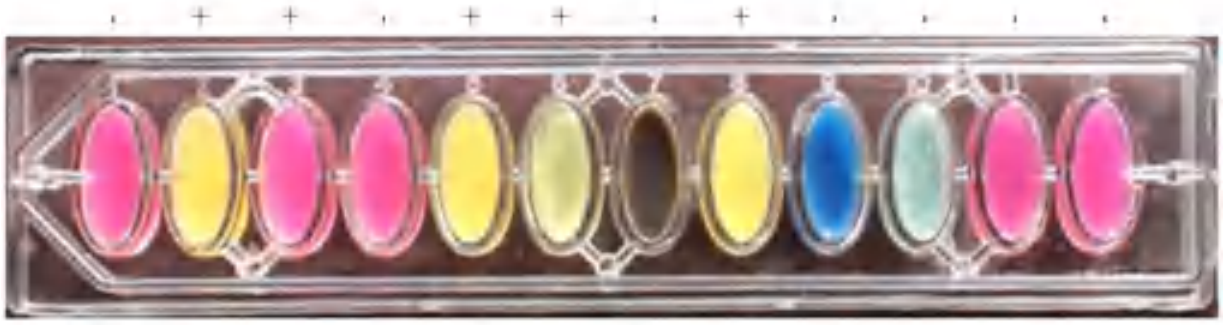
red test, Voges Proskauer's test, Citrate utilization test, Glucose utilization test, Adonitol utilization test, Arabinose utilization test, Lactose utilization test, Sorbitol utilization test, Manitol utilization test, Rhamnose utilization test and Sucrose utilization test. The results have been presented in the table 4.10 and Fig.4.12, Fig 4.13 & Fig. 4.14.

From the results it was found that RSG01 isolate was Methyl red, VP, Glucose, Adonitol and Lactose positive. RSG02 isolate was VP, Adonitol and Manitol positive. But RSG03 was VP, Glucose, Adonitol and Manitol positive.

Table 4.10: Carbohydrate utilization test using Hi-carbo Kit of Himedia

Carbohydrates	**Principle**	**Bacterial Isolates**		
		RSG01	**RSG02**	**RSG03**
Indole	Detects deamination of tryptophan	-	-	-
Methyl red	Detects acid production	+	-	-
Voges Proskauer's (VP)	Detects acetoin production	+	+	+
Citrate Utilization	Detects capability of organism to utilize citrate as a sole carbon source	-	-	-
Glucose	Carbohydrate utilization	+	-	+
Adonitol	Carbohydrate utilization	+	+	-
Arabinose	Carbohydrate utilization	-	-	+
Lactose	Carbohydrate utilization	+	-	+
Sorbitol	Carbohydrate utilization	-	-	-
Manitol	Carbohydrate utilization	-	+	-
Rhamnose	Carbohydrate utilization	-	-	-
Sucrose	Carbohydrate utilization	-	-	-

A: Carbohydrate kit before use (Hi-carbo kit HiMedia)



B: Carbohydrate kit after utilization by bacteria (Hi-carbo kit HiMedia)

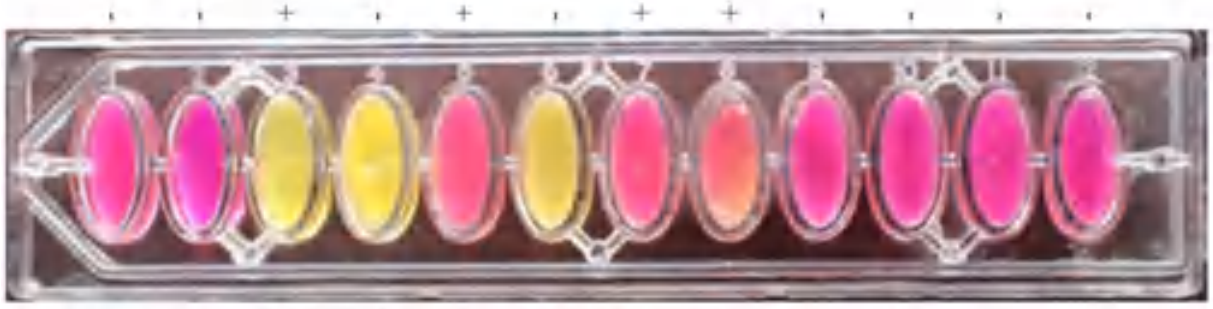
Fig. 4.12: Carbohydrate utilization test of RSG01 isolate of *Ralstonia solanacearum*

A: Carbohydrate kit before use (Hi-carbo kit HiMedia)

B: Carbohydrate kit after utilization by bacteria (Hi-carbo kit HiMedia)

Fig. 4.13: Carbohydrate utilization test of RSG02

A: Carbohydrate kit before use (Hi-carbo kit HiMedia)



B: Carbohydrate kit after utilization by bacteria (Hi-carbo kit HiMedia)

Fig. 4.14: Carbohydrate utilization test of RSG03

4.3.3: Molecular characterization of bacterial isolates and identification.

For molecular identification, bacterial DNA was isolated from all the 26 bacteral isolates. The details of DNA isolation procedures, Quantification of genomic DNA gel electrophoresis and sequencing of the PCR products have been mentioned in details in the materials and methods (Sections 3.8 & 3.9). Phylogenetic analysis procedures have also been described in section 3.10).

At the onset of experiment 16S rDNA primer set was used and the expected amplicons were detected on agarose gels. After this, PCR products of three selected virulent bacterial isolates T6/RSG01, D3/RSG02 and D4/RSG03 were sent to to Xcelris Genomics Ltd. for sequencing. Annotation and BLAST analysis were done and deduced amino acid sequences were submitted to GenBank and necessary accession numbers were procured.

After sequencing the sequence data were analyzed using BLAST at the NCBI website. The sequence data were then analyzed using Clustal W from MEGA version 6.0 software. After BLAST analysis, the nucleotide (nt) sequences were submitted in the GenBank. The details of the identified bacteria and their GenBank Accession nos. were tabulated in table no. 4.11.

Table: 4.11: GenBank accession nos. of 16s rRNA genes received of three virulent pathogenic bacteria of tomato.

Isolate Code	Title	Host	Collection Place	Accession No.
RSG01	*Pseudomonas* sp.T_6 16S ribosomal RNA gene, partial sequence	*Lycopersicon esculentum* Mill.	Siliguri,West Bengal, India	KC237236
RSG01	*Ralstonia solanacearum* strain D_3 16S ribosomal RNA gene, partial sequence	*Lycopersicon esculentum* Mill.	Siliguri, West Bengal, India	KM434237
RSG01	*Ralstonia solanacearum* strain D_4 16S ribosomal RNA gene, partial sequence	*Lycopersicon esculentum* Mill.	Cooch Behar, West Bengal, India	KM434238

 # Molecular identity of the three bacteria by phylogenetic analysis:

For further analysis sequence identity matrix and phylogenetic tree were created by using 16S rRNA region of the three bacteria with that of different *Ralstonia solanacearum* species available in the GenBank. The sequences taken from GenBank were of different countries worldwide.

From the results (Fig. 4.15 and Fig. 4.16) the nucleotide sequence of RSG01 showed sequence similarity with some Indian isolates but was in separately sub clustered. Identity of the bacteria was confirmed as it clustered with *Ralstonia solanacearum*. Sequence of RSG01 (KC237236) of the present study showed closest similarity with Indian isolate KP017457.

The 16S rRNA region of RSG02 and RSG03 clustered together with 99% sequence similarity among them and clustered with Indian isolates. The closest Indian isolate was KM502217. Indian isolates were also clustered with some USA, Thailand and Australian isolates.

 # *fliC* gene based identification:

Ralstonia solanacearum found in xylem vessels are nonmotile, but presence of flagella and motility has been correlated with virulence by several scientists. The flagellum is a filamentous structure made up of about 20,000 copies of flagellin which polymerize into a complex helix structure. Flagellin is produced by *fliC* gene. A specific and sensitive PCR detection method of *Ralstonia solanacearum* using *fliC* gene was established by Schonfeld et al. (2003).

In the present study, it was considered to amplify *fliC* gene of three most virulent bacteria. It was also considered to establish co-relationship, if any, among the three virulent *Ralstonia solanacearum* isolates (RSG01, RSG02 & RSG03) and also to identify the bacteria by nucleotide identity matrix and by phylogenetic tree. For this, *fliC* gene specific primers were used. PCR products were subjected to sequencing and blast analysis. The sequences were submitted to GenBank and accession nos. were received (**Table 4.12**).

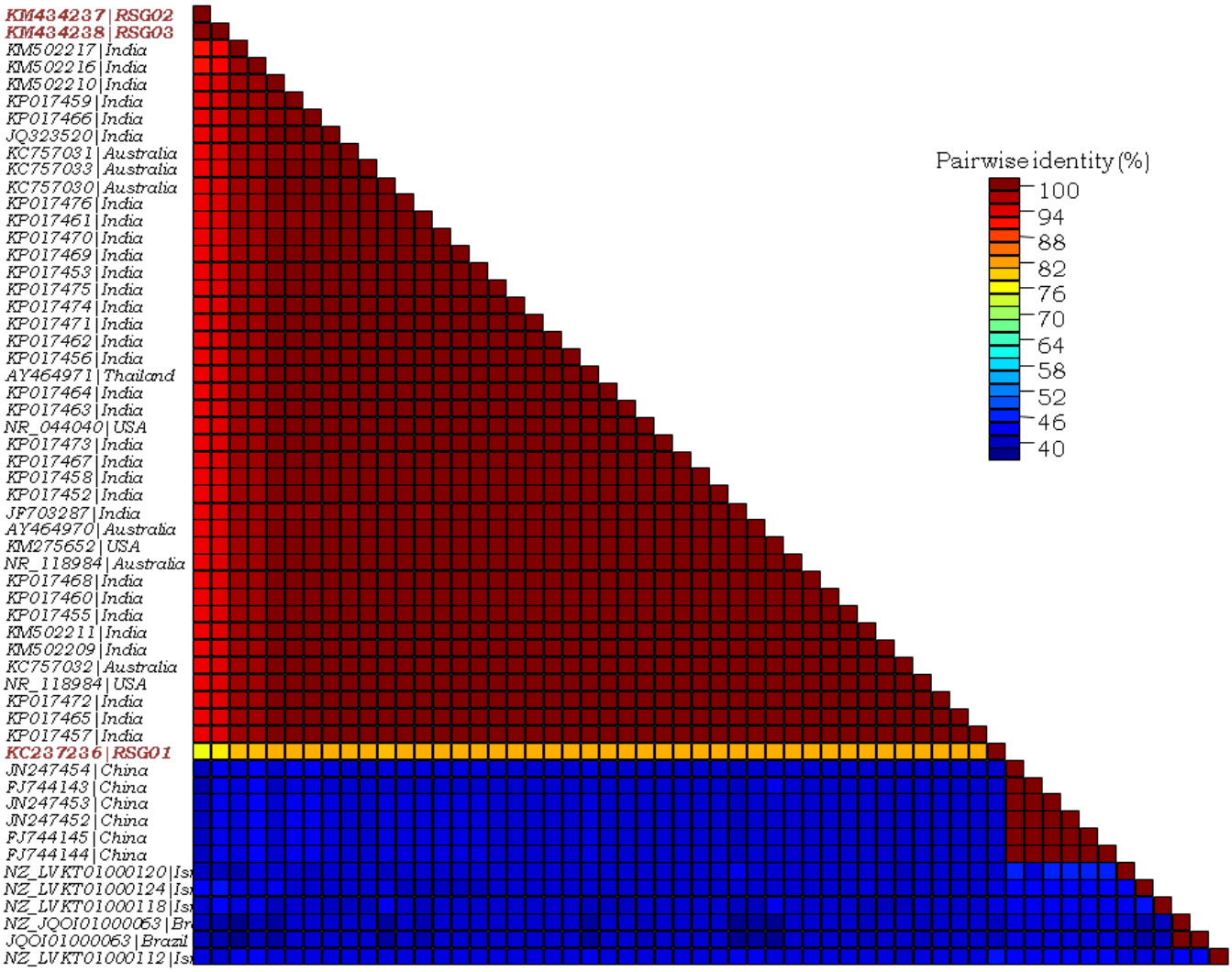


Fig. 4.15: Nucleotide sequence identity matrix of *three Ralstonia solanacearum isolates of the present study and other Ralstonia solanacearum of GenBank following 16s rRNA sequence analysis. Identity percentages are indicated on the right side corner of the matrix.*

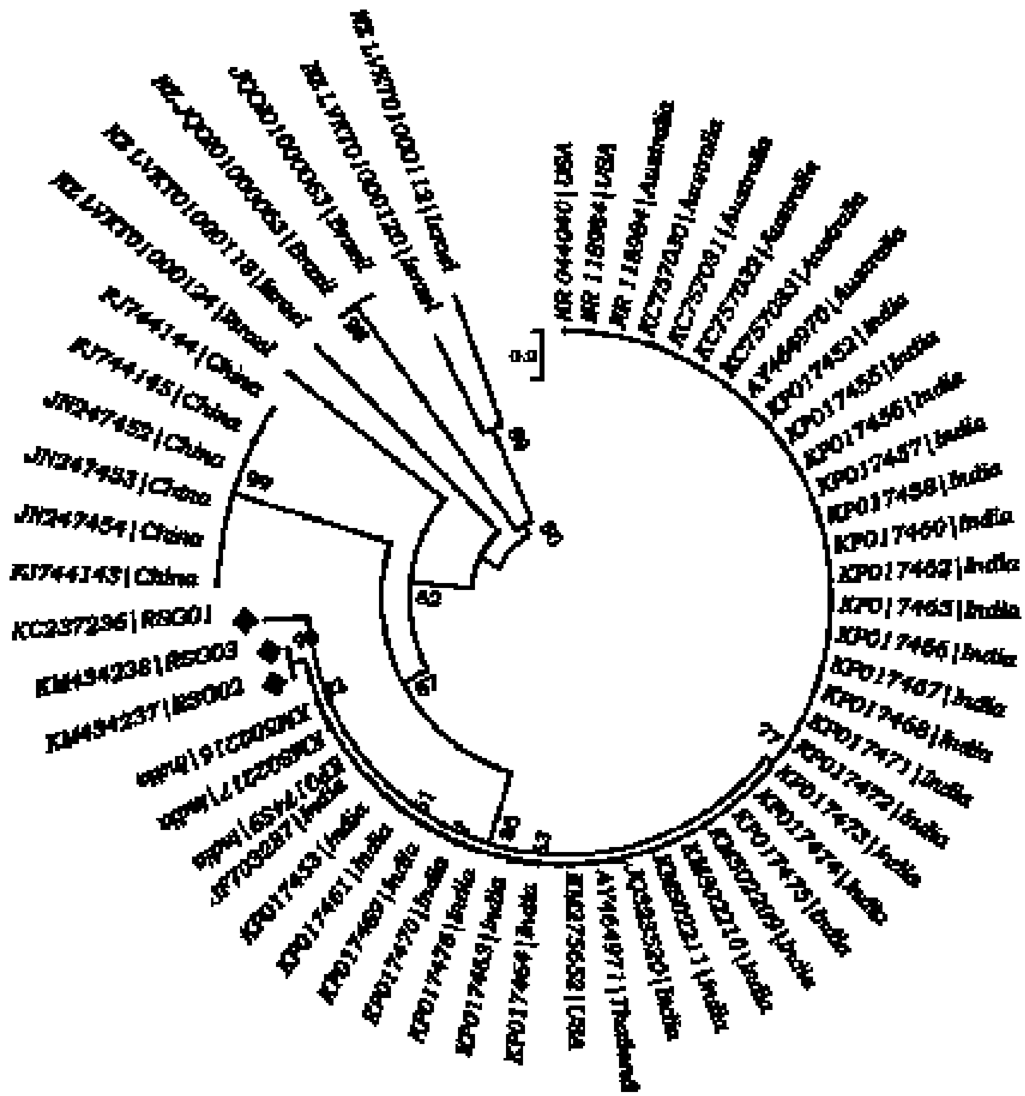


Fig. 4.16: Phylogenetic tree generated by neighbour joining of different *Ralstonia solanacearum. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates) and are indicated if greater than 50. GenBank accession numbers of the bacteria have been indicated at the end of each branch*

Table: 4.12: GenBank accession nos. of *fliC* genes received of three virulent pathogenic bacteria of tomato.

Isolate code	**Title**	**Host**	**Collection Place**	**Accession No.**
RSG01	*Ralstonia solanacearum* strain T_6 flagellin protein (*fliC*) gene, partial cds	*Lycopersicon esculentum* Mill.	Siliguri, West Bengal, India	MG941015
RSG02	*Ralstonia solanacearum* strain D_3 flagellin protein (*fliC*) gene, partial cds	*Lycopersicon esculentum* Mill.	Siliguri,West Bengal,India	MG941016
RSG03	*Ralstonia solanacearum* strain D_4 flagellin protein (*fliC*) gene, partial cds	*Lycopersicon esculentum* Mill.	Coochbehar , West Bengal, India	MG941017

From the results of nucleotide sequence identity and on the basis of phylogenetic tree, constructed based on *fliC* gene (presented in Fig. 4.17 & 4.18 respectively), it was evident that the RSG01 and RSG03 clustered together in a sub group. That group again clustered with RGS02. *fliC* gene sequence of RSG02 isolate was very much close to USA isolates DQ657703 and DQ657701 submitted in GenBank. *fliC* gene sequence of RSG01 and of RSG03 isolate were very much close to Indian isolate KF920693 and Japanese isolate KF275630 as recorded in GenBank.

From the phylogenetic tree of *fliC* gene, RSG01 and RSG03 was very much close to each other but formed a cluster with 98-99 % similarity. 94% similarity was found when the cluster was compared with RSG02 isolate.

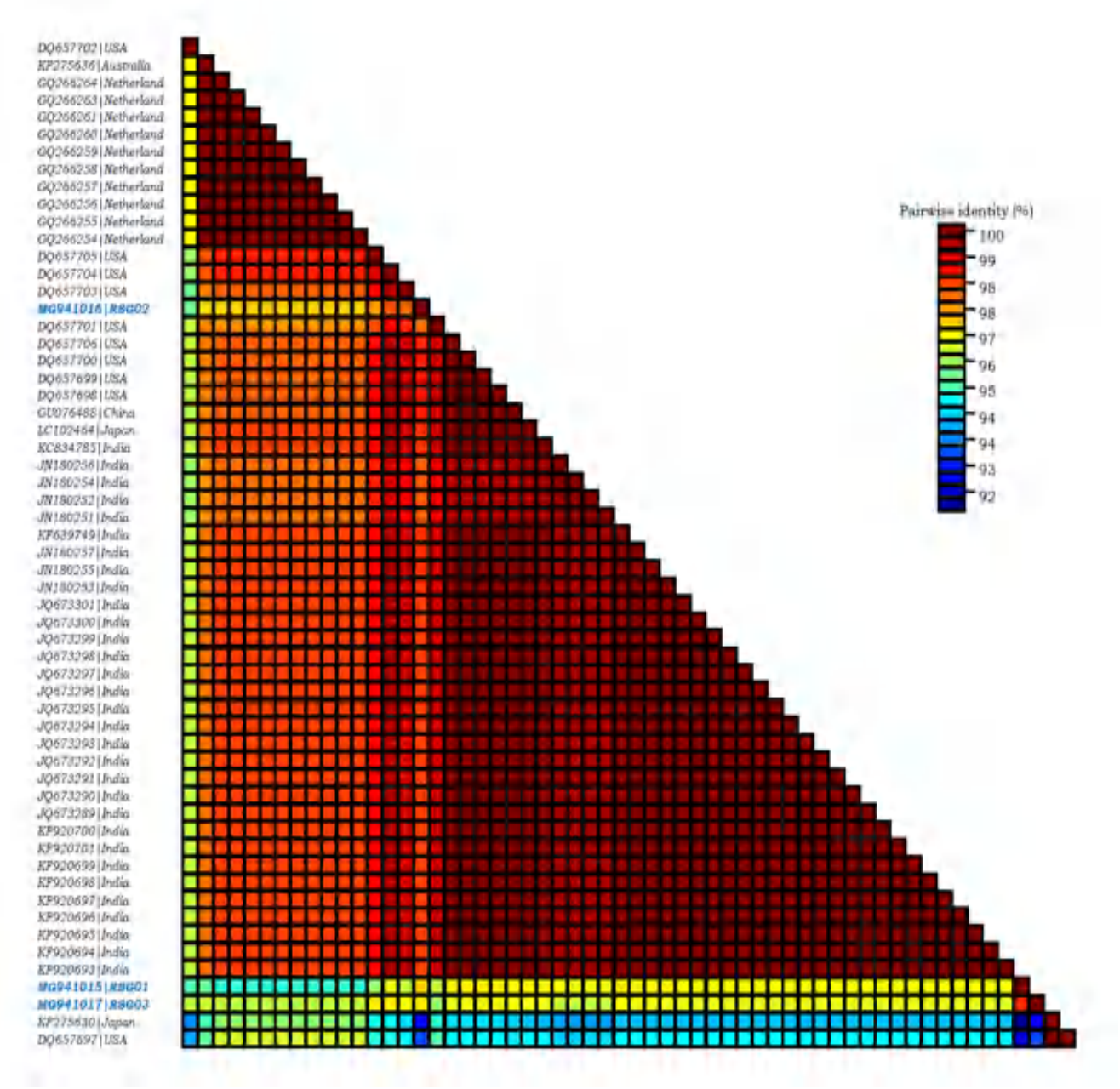


Fig. 4.17: Nucleotide sequence identity matrix of *three Ralstonia solanacearum isolates of the present study and other Ralstonia solanacearum of GenBank based on fliC gene analysis. Identity percentages are indicated on the right side corner of the matrix.*

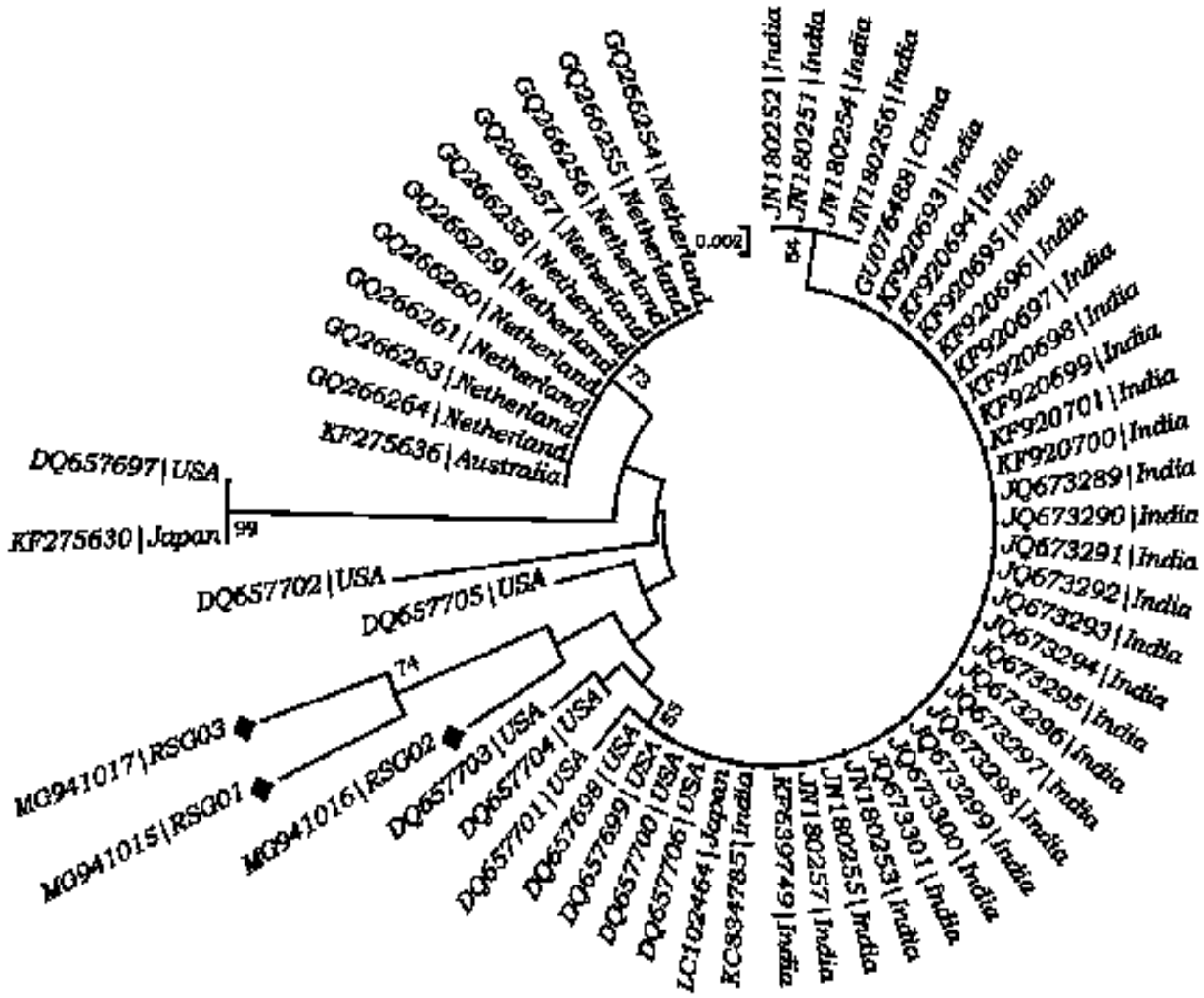


Fig. 4.18: Phylogenetic tree generated by neighbour joining of different *Ralstonia solanacearum based on fliC gene. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates) and are indicated if greater than 50. GenBank accession numbers of the bacteria have been indicated at the end of each branch*

4.4: Chapter IV: Isolation and characterization of antagonistic bacteria from soil and application of antagonistic bacteria for wilt disease management.

Isolation of antagonistic bacteria:

Isolation of antagonistic bacteria were done following the method of Lwin and Ranamukhaarachchi (2006) and described in details in materials and methods

section 3.4.2. All the purified bacteria were subjected to dual culture against *Ralstonia solanacearum* in Nutrient agar medium and were incubated at 30°C up to 96 hours. Any such bacterium found to show antagonism against the *Ralstonia solanacearum* by formation of inhibition zone or growth restriction were considered as antagonistic bacterium.

In the present study three such bacteria were found to be antagonistic against *Ralstonia solanacearum* (isolate RSG01). Those antagonistic bacteria were coded as HS01, HS02 and HS03 and were kept for further experiments. Among the three bacteria HS01 showed best antagonism and restricted growth of *Ralstonia solanacearum* (isolate RSG01) Table 4.13 & Fig. 4.19.

Table 4.13: Growth inhibition of *R. solanacearum* (RSG01) by indigenous bacterium (isolate HS01), isolated from soil of the present study.

Time after inoculation	Breath of the bacterial streak (mm)*	
	Breath of *Bacillus* sp. (Antagonist)	Breath of *R. solanacearum* (Pathogen)
12 h.	13.5	3.7
24 h.	18.0	3.7
48 h.	21.0	3.9
72 h.	24.0	3.9
*Data are mean of three replications		

Morphological, biochemical and molecular characterization.

After isolation and screening of three antagonistic bacteria it was considered to characterize the three bacteria by morphological and biochemical characters. For this different morphological parameters like shape, occurrence, size, spore

formation and colony morphology were studied (Table-14). The procedures of the tests have been discussed in the materials and methods section 3.6.

The biochemical parameters studied were Gram reaction, motility test, growth at different temperatures, indole production, VP test, Pigmentation in nutrient agar, anaerobic growth, catalase and oxidase tests. In addition four different carbohydrates were tested for acid formation. Carbohydrates used

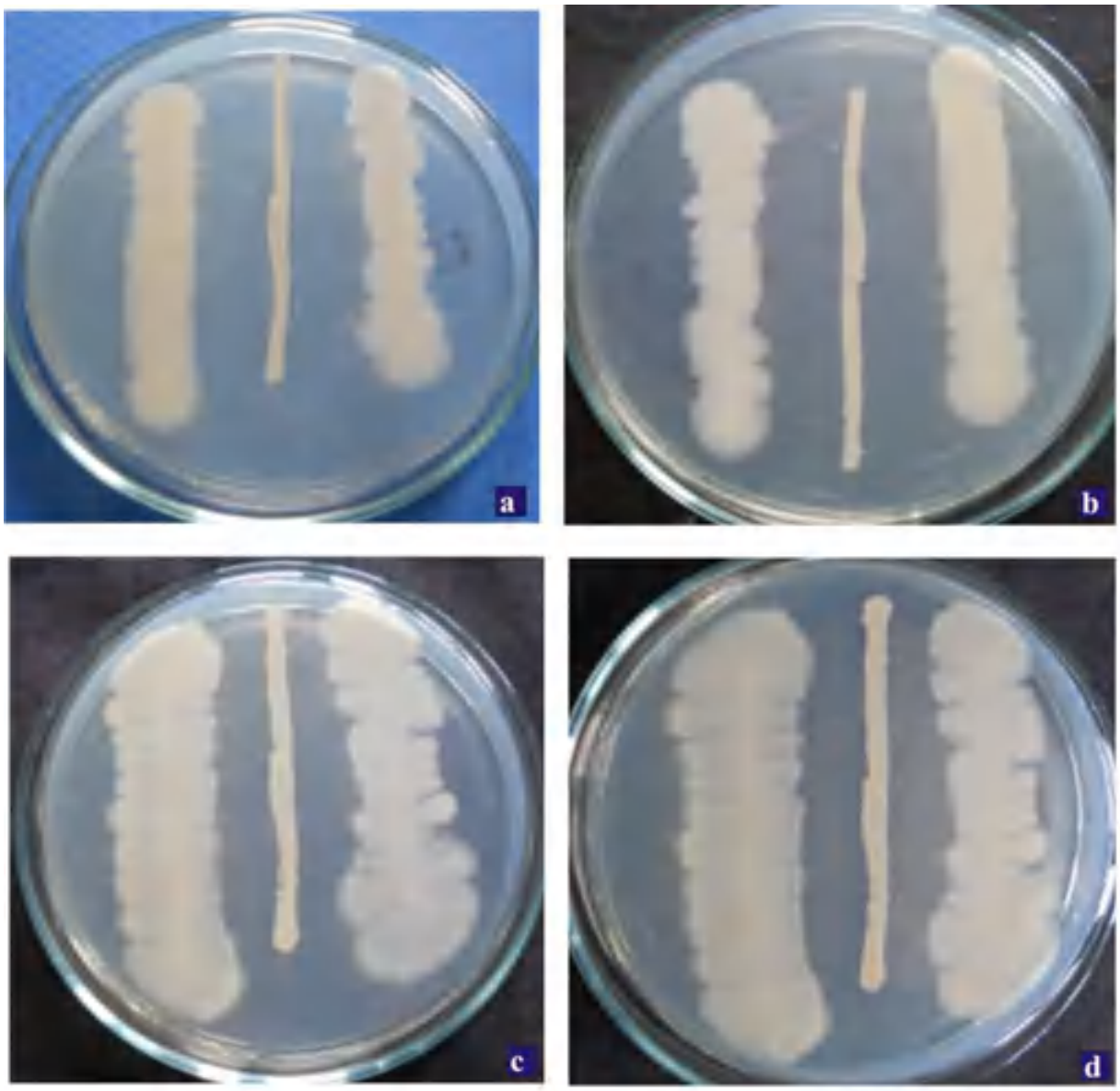


Fig. 4.19: Duel culture test of Ralstonia solanaceurm strain RSG01 against antagonistic bacteria Bacillus sp. Isolated from North Bengal.
a) Observation after 12 hr. b) observation 24 hr. c) observation after 48 hr. d) observation after 72hr.

were from glucose, arabinose, manitol and rhamnose. Details of the experimental procedures have already been discussed in the materials and methods (Section 3.6). The results of the tests mentioned above have been presented in the table 4.15.

Table 4.14: Morphological characterization of three bacterial antagonists HS01, HS02 and HS03 isolated from soil of sub-Himalayan West Bengal

	Bacterial isolates		
	HS01	HS02	HS03
Shape	Rod shaped	Rod shaped	Rod shaped
Occurrence	Single, paired, short chained	Single, paired, short chained	Single, paired, short chained
Size	3-3.5 µm X 1.8-2.2 µm	2.8-3.2 µm X 1.5-2.1 µm	3.0-3.2 µm X 1.9-2.1 µm
Spore	+	+	+
Colony morphology	Ellipsoidal, sub terminal Mucosal surface	Ellipsoidal, sub terminal Mucosal surface	Ellipsoidal, sub terminal Mucosal surface

Table 4.15: Biochemical characterization of three bacterial antagonists HS01, HS02 and HS03 isolated from soil of sub-Himalayan West Bengal

	Bacterial isolates		
	HS01	HS02	HS03
Gram Reaction	+	+	+
Motility	+	+	+
Growth at 25˚C	+	+	+
Growth at 30˚C	+	+	+
Growth at 37˚C	+	+	+
Growth at 42˚C	-	-	-
Indole production	-	-	-
V-P test	-	-	-
Anaerobic growth	-	-	-

Pigmentation in Nutrient agar	-	-	-
Acid from Glucose	+	+	+
Acid from Arabinose	-	-	-
Acid from Manitol	-	-	-
Acid from Rhamnose	-	-	-
Catalase	+	+	+
Oxidase	+	+	+

 # Molecular identification:

On the basis of morphological and biochemical tests performed all the three bacteria were tentatively identified as *Bacillus* sp. As one of the isolate (HS01) showed best antagonism against the most virulent pathogenic isolate (RSG01), it was considered to identify the organism by 16s rRNA study. Following sequence identity and phyllogenetic tree construction the organism (isolate HS01) was identified as *Bacillus cereus.* The sequence of the 16S ribosomal RNA gene (partial) was submitted to GenBank and accession no. of the sequence was procured from GenBank (table 4.16).

Table: 4.16: GenBank accession no. of 16s rRNA genes received

Isolate code	**Title**	**Host**	**Collection Place**	**Accession No.**
HS01	*Bacillus cereus* strain HS01 16S ribosomal RNA gene,partial sequence	*Lycopersicon esculentum* Mill.	Siliguri, West Bengal, India	KC959841

 # Management of bacterial wilt disease of tomato by soil application of antagonists.

After the characterization of the antagonistic bacteria they were considered to be tested for disease management. For this two different types of experiments were performed such as in pots and in Fields. Details of the experimental procedures have been discussed in materials and methods section 3.11.5 & 3.11.6. The results of the experiments have been presented in the tables 4.17 & 4.18.

From the results of table 4.18, it is evident that *Bacillus cereus* isolate HS01 was best among the tested antagonists when applied separately. It could check wilt disease up to 20 days in pots Fig 4.20c.

After the experiment done in pots, it was considered to assess the reduction of disease in fields following bacterization of roots of tomato plants. The results (Table 4.19) of the experiment reveal that in field condition only *Bacillus cereus* (isolate HS01) could check the disease significantly. The other two antagonists could reduce the disease to some extent.

Fig. 4.20: *In vivo* application of bio-control agents in potted plants. a) Control (No treatment done) b) Inoculated with *Ralstonia solanacearum* (Strain RSG01) c) Plants applied with *Trichoderma harzianum* and inoculated by *Ralstonia solanacearum* (Strain RSG01) d) Plants applied with *Bacillus cereus*. and inoculated by *Ralstonia solanacearum* (Strain RSG01)

Table 4.17: Assessment of disease reduction by application of antagonistic bacteria in inoculated tomato plants in pots.

Inoculation status	**Application of antagonist** (100ml broth/1.5 kg soil)	**Wilting index* (days after inoculation)**			
		5d	10d	15d	20d
Set I *Ralstonia solanacearum* Inoculated	Antagonist not applied	-	++	+++	++++
Set II *R. solanacearum* Inoculated	Suspension of isolate HS01 (*Bacillus cereus*)	-	-	-	-
Set III *R. solanacearum* Inoculated	Suspension of isolate HS02	-	-	+	+
Set IV *R. solanacearum* Inoculated	Suspension of isolate HS03	-	-	+	+
Set V *R. solanacearum* Inoculated	Mixed suspension of HS01, HS02 & HS03 (33.33ml each)	-	-	-	+
Set VI (Control) Un-inoculated	Antagonist not applied	-	-	-	-
*Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.					

Table 4.18: Assessment of wilt disease by application of indigenous *Bacillus* sp. by root bacterization process in field condition

Antagonist applied for bacterization	**Inoculation status**	**Wilting index* (days after inoculation)**			
		5d	10d	15d	20d
Tomato roots were dipped into suspension of *Bacillus cereus* (HS01)	Soil inoculation by *R. solanacearum* (RSG01) suspension	-	-	-	-
Tomato roots were dipped into suspension of *Bacillus sp.* (HS02)	Soil inoculation by *R. solanacearum* (RSG01) suspension	-	+	++	+++
Tomato roots were dipped into suspension of *Bacillus sp.* (HS03)	Soil inoculation by *R. solanacearum* (RSG01) suspension	+	+	++	+++
Tomato roots were dipped into Mixed suspension of *Bacillus* sp.(HS01 +HS02+HS03) in equal ratio	Soil inoculation by *R. solanacearum* (RSG01) suspension	-	-	++	++
Control positive Tomato roots were dipped into sterile distilled water	Soil inoculation by *R. solanacearum* (RSG01) suspension	+	++	+++	+++++
Control Tomato roots were dipped into sterile distilled water	Soil un-inoculated	-	-	-	-
*Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21-40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.					

4.5: Chapter V: Management of bacterial wilts by application of antagonistic fungus *Trichoderma harzianum*

4.5.1: *In vitro* control of *Ralstonia solanacearum* with *Trichoderma harzianum*:

Dual culture technique as described in materials and methods (section 3.12.1) was followed to detect the antagonistic potentiality of *T. harzianum* against *R. solanacearum*. Results were noted in table 4.19 and Fig. 4.21. From the results it was evident that *T. harzianum* could check the growth of *R. solanacearum* completely.

Table: 4.19 Growth inhibition of *R. solanacearum* (isolate RSG01) by *Trichoderma harzianum*

Time after inoculation	Growth	
	Growth of Antagonistic fungi	Growth of *R. solanacearum* (Pathogen)
24h	Fungus grown in 50 % area of the Petri plate	Bacterial streak visible but growth was restricted
48 h	Covered the whole Petri plate spores of the fungus was present in a scattered manner.	Bacterial streak visible but growth was restricted.
72 h	Covered the whole Petri plate. Spores visible in 60% area.	Bacterial streak covered by antagonist.
96 h	Covered the whole Petri plate. Spores visible in 95% area.	Bacterial streak covered by antagonist.
120 h	Covered the whole area and spores visible in 100% area.	Bacterial streak covered by antagonist. Bacterial growth completely inhibited.

4.5.2: *In vivo* control of wilt caused by *R. solanacearum* by *T. harzianum*:

After *in vitro* test it was considered to apply *T. harzianum* in whole plants in pot condition in soil. Procedures of the experiment have been discussed in

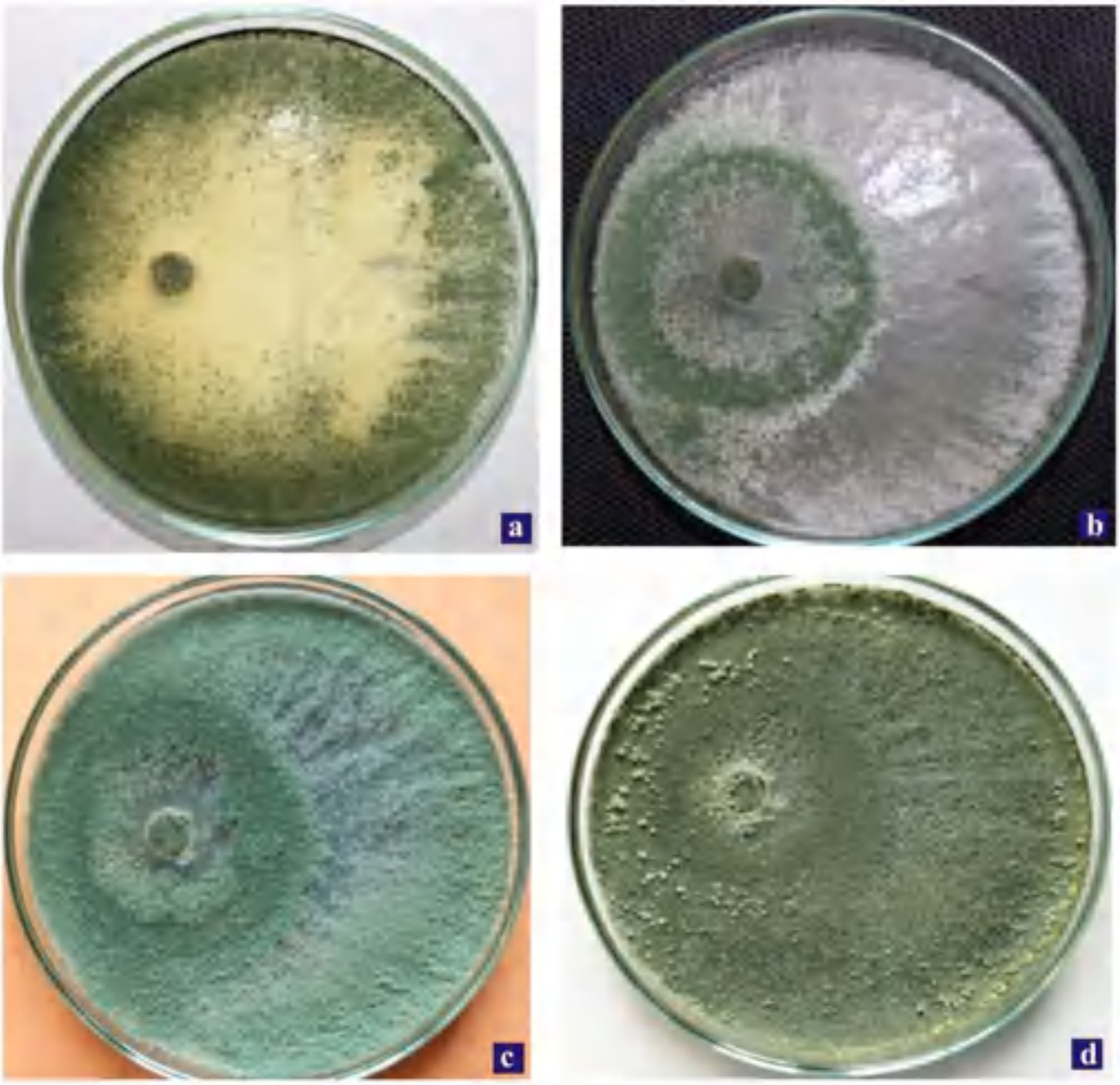


Fig. 4.21: In vitro dual culture test of Ralstonia solanacearum and Tricoderma harzianum a) Observation after 48hr. b) Observation 72 hr. c) Observation 96hr d) Observation after 120 hr.

materials and methods section 3.12.2. and the results have been presented in table 4.20. and Fig. 4.20d.

From the results it was clear that *T. harzianum* could reduce the disease (wilting index) up to 80% in sterilized soil in potted plants.

Table: 4.20: Assessment of disease reduction by application of antagonistic fungus in inoculated tomato plants in pots.

Inoculation status	**Application of antagonist** (100ml spore suspension/1.5 kg soil)	**Wilting index* (days after inoculation)**			
		5d	10d	15d	20d
R. solanacearum inoculated	Antagonist not applied	-	++	+++	++++
R. solanacearum inoculated	Spore suspension of *T. harzianum* applied	-	-	-	-
(Control) Un-inoculated	Antagonist not applied	-	-	-	-

*Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

4.6: Chapter VI: Management of bacterial wilt by application of selected botanicals

From the literature it has been found that some plants possess antibacterial activity. Considering the antibacterial activity some plants were selected to check their potentiality against *R. solanacearum in vitro*. Details of the experimental process have been described in materials and methods (section 3.13) and results of the experiment have been presented in table 4.21, Fig. 4.22 & 4.23.

From the results, it was evident that extract of *Zingiber officinale, Azadirachta indica* and *Camellia sinensis* could inhibit the growth of the *R. solanacearum* significantly.

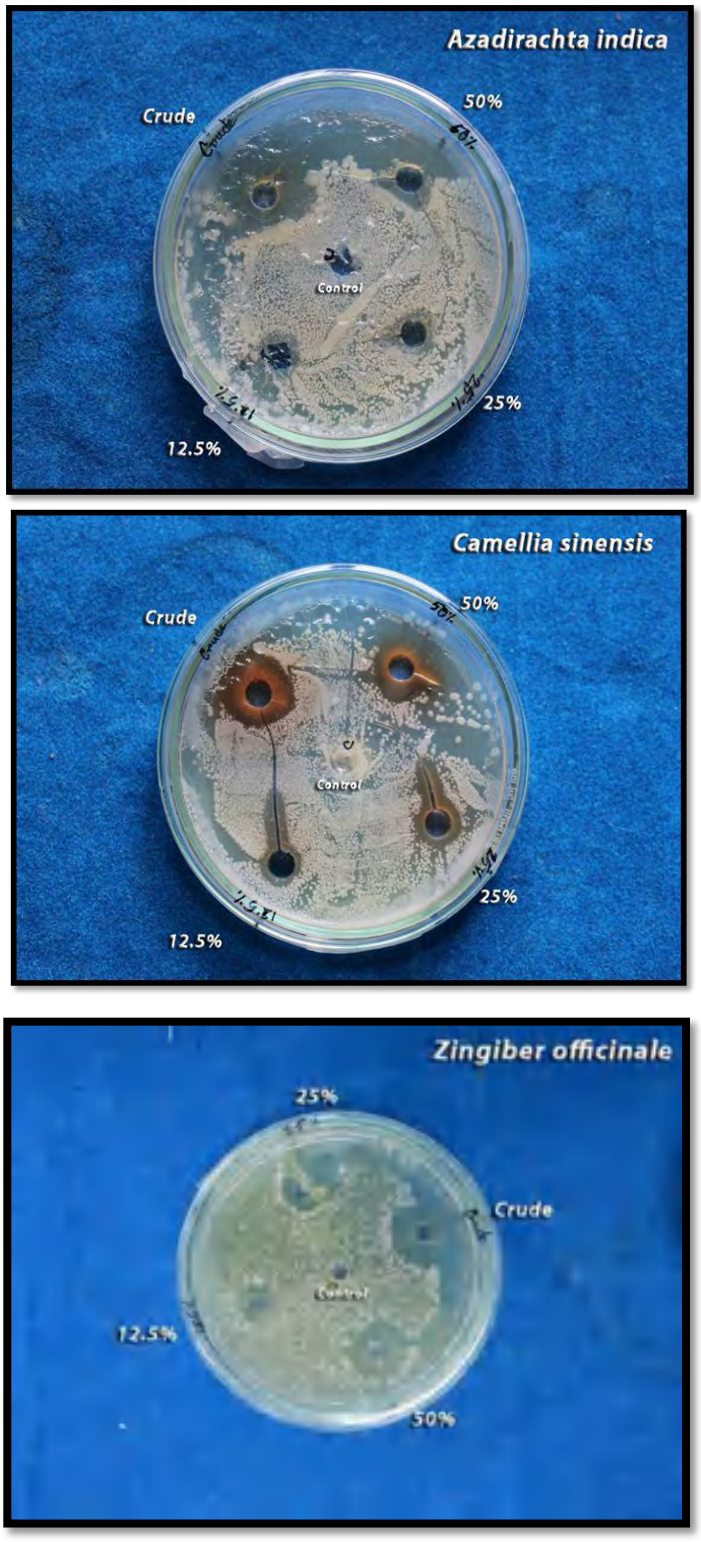


Fig. 4.22 Inhibition of growth of *Ralstonia solanacearum* by *Azadirechita indica*, *Camellia sinensis* and *Zingiber officinale*

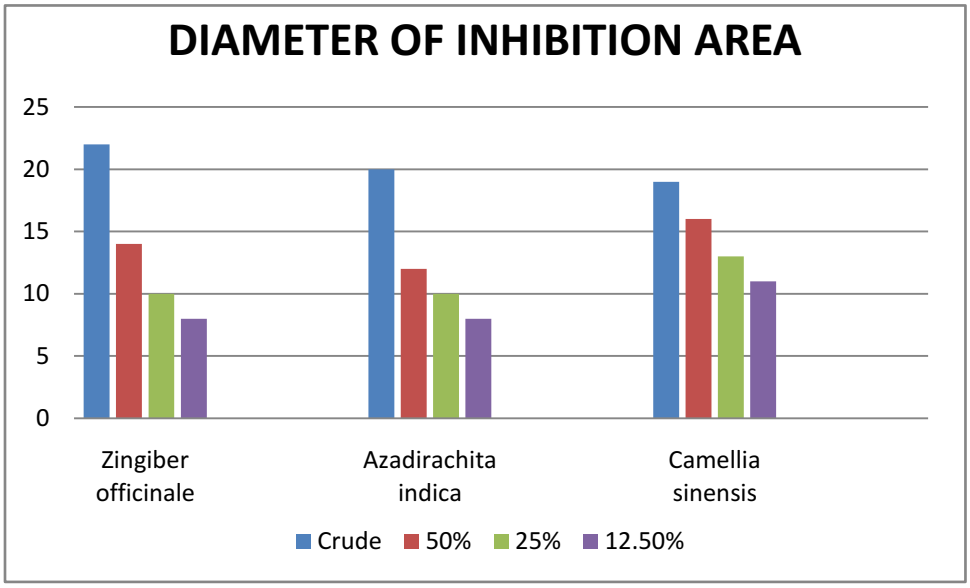


Fig. 4.23. Graphical representation of inhibition of growth of 3 selected botanicals in four different concentrations

Table 4.21: Growth inhibition of *R. solanacearum* by eight different botanicals in four different concentrations

Sl. No.	Plant species tested	Diameter of inhibition zone (mm)			
		Crude	50%	25%	12.5%
1.	*Spermacoce hispida*	15	13	10	7
2.	*Allium sativum*	19	14	11	3
3.	*Zingiber officinale*	22	14	10	8
4.	*Azadirachta indica*	20	12	10	8
5.	*Ocimum Sanctum*	18	15	10	7
6.	*Cinamomum tamala*	15	12	10	8
7.	*Camellia sinensis*	19	16	13	11
8.	*Thuja occidentalis*	0	0	0	0

Crude = 1g leaf/ml extract concentration; Data are mean of three replications

After the *in vitro* studies leaf extract of *Camellia sinensis* were mixed with soil of potted tomato plants. Procedures of the experiment have been discussed in materials and methods (section 3.13.) and the results have been presented in table 4.22.

From the results it was evident that *Camellia sinensis* leaf extract could successfully control *R. solanacearum* caused wilt in tomato in treated plants in comparison to untreated-inoculated plants, where about 40 to 60% wilting was observed (Table 4.23).

Table: 4.22: Assessment of disease reduction by application of tea leaf extract in inoculated tomato plants in pots.

Inoculation status	Application of leaf extract (100ml extract/1.5 kg soil)	Wilting index* (days after inoculation)			
		5d	10d	15d	20d
R. solanacearum inoculated	Leaf extract not applied	-	++	+++	++++
R. solanacearum inoculated	Leaf extract of *C. sinensis* applied	-	-	-	-
(Control) Un-inoculated	Antagonist not applied	-	-	-	-

*Wilting index was calculated on the basis of a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21 -40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants.

4.6: Chapter VII: Induction of defense-related enzymes in tomato plants by abiotic inducers and studies on some defense related enzymes.

Among the chemical inducers of defense found in literature three inducers (BABA, SA and ABA) were selected for the present study the inducers were used for the purpose of defense induction in tomato against *Ralstonia solanacearum* (isolate RSG 01). Induction of resistance in tomato plants was evaluated by computing the mean foliar disease index based on visual observation. Procedures of the treatment and enzyme estimation process have been discussed in the materials and methods (sections 3.14.1). Concentration of the chemical inducers used in the induction process of tomato (cv. Pkm-1) was 10^{-3} m.

Four different sets of experimental plants were 'untreated-uninoculated (control)', 'untreated-inoculated (control)', 'treated-uninoculated' and 'treated-inoculated'. Wilting index was determined for all the four sets. Results showed that SA reduced wilting index significantly in comparison to control (Table. 4.24). After 6 days of inoculation, wilting symptoms appeared in untreated-inoculated plants but not in those treated with any of the chemical inducers. After 6 days, the untreated plants showed wilting index of about 18% (Graded

as + in the table), thereafter, it increased in 9 days and 12 days (showing about 50% of wilting; graded as +++). BABA treated plants showed wilting (about 30%, graded as ++) after 12 days. ABA treated plants showed wilting of plants after 9 days of inoculation. There was no visual change in the untreated-uninoculated plants. From the results it was evident that, the efficacy of SA was best to reduce wilting of tomato in plants against *R. solanacearum* isolate RSG01, in comparison to BABA and ABA.

Table: 4.23: Assessment of disease reduction in chemical inducer pre-treated tomato plants following inoculation of *R. solanacearum* in pot grown tomato plants.

Application of chemical inducers & Inoculation status	**Wilting index* (days after inoculation)**			
	3d	6d	9d	12d
Untreated-Uninoculated (control)	-	-	-	-
Untreated-Inoculated (control)	-	+ (18%)	++ (30%)	+++ (50%)
BABA treated-Inoculated	-	-	-	+ (15%)
SA treated-Inoculated	-	-	-	-
ABA treated- Inoculated	-	-	+ (12%)	+ (20%)

*Wilting index was calculated on the basis of 5visual observation based on a 5 point scale. - = no disease, + = drooping of the leaves up to 20%, ++ = drooping of plants between 21-40%, +++= drooping of plants between 41-60%, ++++= 61-80%, +++++ = 100% drooping and wilting of plants. Data are mean of three replications; BABA= β-amino butyric acid; SA= Salicylic acid; ABA= Abscisic acid.

4.6.1: Activity of phenylalanine ammonia lyase on application of abiotic inducers

Results of PAL activity have been presented in tables 4.24 and Fig. 4.24. Results reveal that SA treated plants showed highest PAL activity (2.84 µmol min^{-1} g^{-1} fresh weight tissue) after one day of inoculation. When simultaneous inoculation and treatment was done with SA, PAL activity became more (3.2

μmol min^{-1} g^{-1} fresh weight tissue) after 3 days of inoculation. From the results the increase was found to be about 2 times. BABA and ABA treated plants showed comparatively less PAL activity. BABA treated plants showed 2.60 (μmol min^{-1} g^{-1} fresh weight tissue) activity after 3 days of inoculation. BABA treated and inoculated plants showed increased activity (2.80 μmol min^{-1} g^{-1} fresh weight tissue) after 3 days of inoculation. ABA treated plants also induced PAL activity but much lower than SA treated plants. ABA treated and ABA treated-inoculated plants showed activity of the enzyme respectively 2.0 and 2.3 (μmol min^{-1} g^{-1} fresh weight tissue) respectively.

Table 4.24: PAL activity in tomato roots (variety PKM-1) pretreated with abiotic inducers and inoculated by *Ralstonia solanacearum*

Treatment	PAL activity* (μmol $min^{-1}g^{-1}$ fresh weight tissue) (Days after inoculation)					
	0	**1**	**2**	**3**	**4**	**5**
BABA	1.62 ±0.03	2.24 ±0.06	2.50 ±0.04	2.60 ±0.05	2.40 ±0.04	2.40 ±0.04
BABA + I	1.63 ±0.04	2.3 ±0.05	2.7 ±0.06	2.8 ±0.04	2.7 ±0.03	2.6 ±0.03
SA	1.62 ±0.03	2.84 ±0.06	2.80 ±0.05	2.50 ±0.06	2.40 ±0.03	2.30 ±0.05
SA+ I	1.63 ±0.02	2.62 ±0.02	3.0 ±0.04	3.2 ±0.02	3.0 ±0.04	2.8 ±0.02
ABA	1.62 ±0.04	1.84 ±0.04	1.90 ±0.03	2.0 ±0.05	2.0 ±0.03	2.0 ±0.04
ABA+ I	1.63 ± 0.03	1.86 ±0.03	2.2 ±0.06	2.3 ±0.03	2.2 ±0.05	2.1 ±0.03
Inoculated	1.62 ± 0.02	1.63 ±0.04	1.67 ±0.02	2.0 ±0.04	2.18 ±0.03	2.29 ±0.06
Control	1.62	1.60	1.62	1.62	1.63	1.63

	±0.02	±0.03	±0.02	±0.03	±0.02	±0.04
*Data are mean of three replications; Data after ± indicate standard error values. **Abbreviations**: BABA= β-amino butyric acid; SA= Salicylic acid; ABA= Abscisic acid; I=Inoculated						

4.6.2: Activity of Peroxidase on application of abiotic inducers

Results of PO activity have been presented in tables 4.25 and Fig. 4.25. Results reveal that SA treated and inoculated plants showed highest PO activity (31 units (ΔA_{420} min^{-1} g^{-1} fresh weight tissue) after three days of treatment and inoculation. When treatment was done with SA, PO activity was 30 units (ΔA_{420} min^{-1} g^{-1} fresh weight tissue) after 3 days of inoculation. From the results the increase in activity was found to be about 2 fold. BABA treated plants showed 24 unit PO activity (ΔA_{420} min^{-1} g^{-1} fresh weight tissue) activity after 3 days of inoculation. But BABA treated-inoculated plants showed about two fold increase in activity (29 ΔA_{420} min^{-1} g^{-1} fresh weight tissue) after 3 days of inoculation. ABA treated plants also induced PO activity but at a much lower rate than SA and BABA treated plants.

Table 4.25: PO activity in tomato roots (variety PKM-1) pretreated with abiotic inducers and inoculated by *Ralstonia solanacearum*

Treatment	Peroxidase activity [ΔA_{420} min^{-1} g^{-1} fresh weight tissue] 1 unit = 0.001 absorbance (days after inoculation)					
	0	1	2	3	4	5
BABA	16 ±0.3	20 ±0.4	23 ±0.6	24 ±0.3	23 ± 0.4	22 ±0.5
BABA + I	16 ±0.2	24 ±0.3	28 ±0.7	29 ±0.5	29 ±0.5	28 ±0.3
SA	15 ±0.5	25 ±0.5	27 ±0.6	30 ±0.5	28 ±0.3	27 ±0.6
SA+ I	16 ±0.4	27 ±0.3	29 ±0.5	31 ±0.4	29 ±0.7	28 ±0.2
ABA	15	18	20	22	22	21

	±0.3	±0.8	±0.5	±0.8	±0.7	±0.4
ABA+ I	15 ±0.4	19 ±0.3	21 ±0.4	23 ±0.3	24 ±0.5	23 ±0.6
Control	15 ±0.3	16 ±0.2	15 ±0.5	15 ±0.3	15 ±0.4	16 ±0.2
Inoculated	15 ±0.2	16 ±0.1	18 ±0.3	19 ±0.3	22 ±0.6	23 ±0.2

*****Data are mean of three replications; Data after ± indicate standard error values.
Abbreviations: BABA= β-amino butyric acid; SA= Salicylic acid; ABA= Abscisic acid; I=Inoculated

4.6.3: Activity of Polyphenol oxidase (PPO) on application of abiotic inducers

Results of PPO activity have been presented in tables 4.26 and Fig. 4.26. Results reveal that SA treated and inoculated plants showed highest PPO activity of 5.6 after four days of treatment and inoculation. When treatment was done with SA, PPO activity was 4.9 after 3 days of inoculation. In case of PPO also more than two fold increase in activity was experienced in cases of SA treated plants. BABA treated plants showed 3.49 unit PPO activity after 4 days of inoculation. After similar period of inoculation BABA treated-inoculated plants showed 3.83 unit activity. ABA treated plants also induced PPO activity but at a much lower rate than SA treated plants.

In conclusion, it may be stated that SA is a potential defense inducer and can control bacterial wilt disease of tomato significantly.

Table 4.26: PPO activity in tomato roots (variety PKM-1) pretreated with abiotic inducers and inoculated by *Ralstonia solanacearum*

Treatment	Polyphenol oxidase activity= K x (ΔA Min^{-1}) µmol Min^{-1} g^{-1}fresh wt. tissue (K= 0.272 for polyphenol oxidase) (Days after inoculation)					
	0	**1**	**2**	**3**	**4**	**5**
BABA	2.74 ±0.03	3.21 ±0.02	3.43 ±0.05	3.50 ±0.03	3.49 ±0.06	3.40 ±0.03
BABA + I	2.73 ±0.02	3.32 ±0.05	3.67 ±0.03	3.82 ±0.04	3.83 ±0.04	3.80 ±.04
SA	2.72 ±0.03	4.34 ±0.04	4.86 ±0.02	4.9 ±0.05	4.86 ±0.06	4.54 ±0.01
SA+ I	2.72 ±0.02	4.36 ±0.03	4.93 ±0.04	5.5 ±0.06	5.6 ±0.03	5.4 ±0.04
ABA	2.72 ±0.01	2.94 ±0.04	3.1 ±0.06	3.2 ±0.03	3.3 ±0.05	3.4 ±0.02
ABA+ I	2.73 ±0.01	3.25 ±0.05	3.4 ±0.03	3.5 ±0.4	3.44 ±0.05	3.34 ±0.05
Inoculated	2.73 ±0.02	2.74 ±0.01	2.78 ±0.03	2.83 ±0.03	3.22 ±0.05	3.34 ±0.04
Control	2.73 ±0.02	2.73 ±0.04	2.72 ±0.02	2.71 ±0.05	2.72 ±0.04	2.74 ±0.03

*****Data are mean of three replications; Data after ± indicate standard error values.

Abbreviations: BABA= β-amino butyric acid; SA= Salicylic acid; ABA= Abscisic acid; I=Inoculated

Chapter-5

DISCUSSION

Tomato is one the popular vegetable crop grown throughout world. Presently tomatoes are grown round the year in India. Northern plains of West Bengal (popularly known as sub-Himalayan West Bengal) produce large quantity of tomato. Among the seven districts of North Bengal three districts are famous for tomato production. Those three districts are Coochbehar, Uttar-Dinajpur and Dakshin-Dinajpur. Several diseases of the crop have been reported from this part. Although most of the diseases are caused by fungi but substantial loss of crops have been reported by bacterial wilt caused by *R. solanacearum* (Windhan and Alan, 2003). Infection in the plants mainly occurs through root-to-root transmission and movement of the contaminated soil (Gopalakrishnan et al., 2014). Swimming motility of *R. solanacearum* allows it to find suitable host and disperse effectively (Kersten et al., 2001). *R. solanacearum* block the water transportation path i.e. the xylem. Although *R. solanacearum* mostly affects solanaceous crops (Alvarez et al. 2008) but a large number of crops and some ornamental plants including tomato are being affected by *R. solanacearum* (Elphinstone, 2005; Hayward, 1994; Fegan and Prior, 2005). Mondal et al. (2011) reported isolation of *Ralstonia solanacearum* from several plants including tomato from West Bengal.

The farmers of the present study area depend mostly on synthetic pesticides for the disease management. Synthetic pesticides alter the natural soil-microflora and also pollute the environment (Al-samarrai et al., 2012; Riah et al, 2014: Mahmood et al. 2016). The residual chemicals of the crop also create health hazard to us. A large number of research works have been done on 'Tomato-Ralstonia' interaction throughout the world (Overbeek et al. 2002; Dannon and Wydra, 2004; Yao and Allen, 2007; Raja et al. 2016; Wang et al. 2019). But very less number of works is available in literature from the present study area.

Considering the above the present work was planned to isolate and identify the bacterial wilt pathogens from the present study area and management of the disease by eco-friendly ways. Identification of the isolated

pathogens has been done by standard morphological, biochemical and molecular methods. Management of the disease has been done by bio-control agents, botanicals and also by induction of inherent resistance by application of inducer chemicals.

Fifty different places/locations of three districts of sub-Himalayan West Bengal (Coochbehar, Uttar Dinajpur and Dakshin Dinajpur) were surveyed for occurrence of the disease in tomato. On the basis of preliminary survey 26 different locations were found to be prone to bacterial wilt disease. Ten locations of Haldibari and three locations of Ghoksadanga both of Coochbehar district were found to show severe bacterial wilt disease symptoms in the cultivated tomatoes. Eight locations of Balurghat of Dakshin-Dinajpur district and four locations of Durgapur of Uttar-Dinajpur district were also found to show severe wilt symptoms during survey. Mondal (2014) reported isolation of *Ralstonia solanacearum* from different parts of West Bengal and described it as a severe pathogen of tomato.

R. solanacearum has been found to be one of the main constraints in the field of agriculture as it causes wilt disease by blocking the water transportation path i.e. the xylem. A large number of crops and some ornamental plants are being affected globally though bacterial wilt caused by the most devastating phytopathogen called *Ralstonia solanacearum* (Elphinstone, 2005; Hayward, 1994; Fegan and Prior, 2005). *Ralstonia solanacearum* mostly affects solanaceous crops (Alvarez et al. 2008).

The plants which showed bacterial ooze in the ooze test were considered for isolation and characterization. Out of the 26 bacterial isolates ten isolates was collected from Haldibari of Coochbehar district. Three isolates were found from Ghoksadanga, also from Cooch Behar district. Four isolates were found from Durgapur region of Uttar Dinajpur District. Nine bacterial isolates were also found from the Balurghat of Dakshin Dinajpur. All the 26 isolated bacteria were assigned isolate codes and were subjected to confirmation of Koch's

postulations based on pathogenicity tests. From the pathogenicity test results 3 bacteria were found to be highly pathogenic to moderately high pathogenic. Thirteen bacteria was pathogenic and 10 bacteria were weakly pathogenic. Three most virulent isolates were subjected to molecular studies and were reassigned codes as RSG01, RSG02 and RSG03 before submission of gene sequences to GenBank. Chaudhury and Rashid (2011) isolated five samples from seven fields of Pakisthan. They selected the bacteria on the basis of fluidal nature of the colony. Our isolates were also showed fluidal nature in colonies. Thus our preliminary identification was in agreement with that of Chaudhury and Rashid (2011). Sebedi et al. (2014) isolated *Ralstonia solanacearum* from seven different fields of Ghana on the basis of ooze collection. Ooze test based isolation of bacteria were also done by Mondal (2014). Our isolated strains also showed streaming of ooze in water. Thus our strains isolated were in the line of some previous workers.

Dutta and Rahman (2012) screened several tomato varieties for determining their nature of resistance and susceptibility against *Ralstonia solanacearum* in Assam. They found one variety as highly resistant, four varieties as moderately resistant, four varieties as moderately susceptible and two varieties as highly susceptible. In the present study, four varieties were tested for their resistance and susceptibility against the most virulent pathogen *Ralstonia solanacearum* (RSG01) by pathogenicity test. Among the varieties (PKM-1, Vaishali, Rupali and Rashmi) tested 'PKM-1' was most susceptible and variety 'Rashmi' was least susceptible against *Ralstonia solanacearum* (isolate RSG01). Varieties 'Vashali' and 'Rupali' were also susceptible but less susceptible than 'PKM-1' variety. Kumar et al (2018) screened 11 lines/varieties of tomato for resistance. James and Mathew (2015) reported that PKM 1 was susceptible against their *Ralstonia solanacearum* isolates in Kerala. Thus present finding of susceptible variety PKM1 is in agreement with that of James and Mathew (2015).

Morris and Moury, (2019) reported that pathogens tend to infect plants that are closely related. This is due to the phylogenetic distance between plant taxa. Closely related plant taxa share a similar environment that is suitable to pathogens. Non host resistance in plants usually depends on constitutive defense traits or pattern recognition receptors (PRRs) along with elicitors of pathogen (Gonzalez et al. 2010, Schultze-Lefert and Panstruga, 2011). In the present study limited Host range test was conducted in some solanaceous plants to check infectivity of the most virulent isolate of tomato to three different solanaceous plants such as potato (*Solanum tuberosum* cv. Kufri Jyoti), Brinjal (*Solanum melongena* cv. Muktakeshi) and Chilli (*Capsicum frutescens* cv. Kull Lanka). Potato showed mild susceptibility but Brinjal and Chilli plants did not show any disease symptoms when inoculated with present isolate of *R. solanacearum*. Thus present virulent strain of tomato is weakly pathogenic to potato and is nonpathogenic to brinjal and chilli. Our results are in agreement with the explanations of resistance and susceptibility of closely related hosts and non host as given by previous workers. Colony morphology of the 26 isolated *Ralstonia solanacearum* was mostly smooth, white and fluidal. All the isolated bacteria were non-spore producing, rod shaped and occurred in single or in pairs. In broth all the cultures were turbid with pellicle and sediments. Pawaskar et al. (2014) showed that the colonies of *R. solanacearum* on nutrient agar medium were smooth circular, raised and dirty white and opaque. Similar results were also found by Stanford and Wolf (1917), Khetmalas (1984) and Tahat and Sijam (2010).

Colony morphology of the three most virulent isolates was also studied on CPG medium. The shape of colonies of the three most virulent isolates (RSG01, RSG02 and RSG03) was irregular and round. Colour of the colonies became reddish to deep red but surface was smooth and milky. Rudrappa, et al. (2016), studied cultural and biochemical characters of *Ralstonia solanacearum* causing bacterial wilt in tomato. *Ralstonia solanacearum* require motility for invasive virulence in tomato (Trans-Kersten et al., 2001). In the

present study all the isolated bacteria showed their motile nature in motility medium. The motility positive nature of the isolated bacteria thus proved to be virulent. Characterization of *Ralstonia solanacearum* isolates using biochemical, cultural, molecular methods and pathogenicity tests were also done by Sharma and Singh (2019).

From the scanning electronic microscopic (SEM) figures, the surface topography of the three virulent isolates (RSG01, RSG02 and RSG03) was more or less smooth with some depressions. The size and shape of the three bacteria were also determined from the SEM figures.

Heim et al., (2002) stated that starvation survival state of *Ralstonia solanacearum* is completely different from that of active growth state. Under prolonged starvation condition, the change of shape of bacteria to round cell and reduction in its size were considered as their strategies to survive in oligotrophic environment (Novitsky and Morita, 1976; Rollins and Colwell, 1986; Ruiz et al., 2001). In the present study, shape of bacteria was elongated in size. Thus the present isolates are in active growth phase. The size is also similar with that of a standard *R. solanacearum* cell.

The induction of viable and non-culturable state named as the VBNC state is one of the adaptation in the oligotrophic state (Oliver, 2005; Roszak and Colwell, 1987). *R. solanacearum* acquires VBNC state after being exposed to copper (Grey and Steck, 2001). In the present work, no bacterial isolates were in VBNC state as all the isolates could grow in medium.

On the basis of Gram reaction and biochemical tests several scientists have identified *Ralstonia* solanacearum (Rahaman, 2010; Pawaskar et al. 2014; Sharma, 2018; Rudrappa et al. 2016). Similarly in the present study, on the basis of Gram reaction and biochemical tests, all the 26 isolated pathogens were identified as Gram negative *Ralstonia* species. The identification up to genus level was suggested by Trigiano et al. (2004). Although all the 26 bacteria were *Ralstonia* but there are minor differences in biochemical tests.

Carbohydrate utilization tests are important for biovar separation of *Ralstonia solanacearum*. Several working groups have separated biovars on the basis of carbohydrate utilization capabilities of different isolates of *R. solanacearum* (Huang et al. 2012; Popoola et al. 2015; Khasabulli et al. 2017). In the present study, from the carbohydrate utilization tests (conducted for the 3 most virulent isolates) it was found that RSG01 isolate was Methyl red, VP, Glucose, Adonitol and Lactose positive. RSG02 isolate was VP, Adonitol and Manitol positive. But RSG03 was VP, Glucose, Adonitol and Manitol positive. This also indicates presence of minor differences among the virulent isolates in carbohydrate utilization status. Findings of the present study are in agreement with that of previous workers and the three virulent varities are of three different biovars.

There are several methods which are routinely used by the scientists for identification of the organisms. Some of them are 1) isolation on semi-selective medium (Kelman, 1954). 2) the methods of Nesmith and Jenkins, (1979) and Engelbrecht, (1994) 3) Serological methods (ELISA or immunofluorescence, (Janse, 1988, Robinson-Smith et al., 1995; Rajeshwari et al, 1998; van der Wolf et al, 2000; Priou et al., 2010) 4) Pathogenicity tests on host plants McCarter et al., 1969, Graham and Lloyd, 1978. But most of the recent studies show PCR based identification of the bacterial pathogens. From the literature it is evident that several scientists have identified *Ralstonia solanacearum* by PCR based molecular techniques followed by BLASTn analysis and phylogenetic analysis (Garcia et al. 2013; Fonseca et al. 2013; Abdurahaman et al. 2016, 2017; She et al. 2017; Kyaw et al. 2019). In the present study 26 bacterial isolates were subjected to PCR amplification of 16S rRNA gene and the expected amplicons were detected on agarose gels. All the bacteria tested were PCR positive indicating their identity as *Ralstonia solanacearum*. The PCR products of three selected virulent bacterial isolates were annotated and analysed by BLASTn and amino acid sequences were submitted to GenBank and necessary accession numbers were procured. The nucleotide sequence of

RSG01 showed sequence similarity with some Indian isolates but was sub - clustered separately. Identity of the bacteria was confirmed as it clustered with *Ralstonia solanacearum*. Sequence of RSG01 (Accession no. KC237236) of the present study showed closest similarity with one Indian isolate (Accession no. KP017457).

The nucleotide sequence of RSG02 and RSG03 clustered together with 99% sequence similarity among them and clustered with Indian isolates. Accession no. of the closest Indian isolate was KM502217. Indian isolates were also clustered with some USA, Thailand and Australian isolates.

A specific and sensitive PCR detection method of *Ralstonia solanacearum* using *fliC* gene was established by Schonfeld et al. (2003). Kubota et al. (2008) also studied *fliC* gene by LAMP method. Bergsma-Vlami et al. (2018) studied different genes of *Ralstonia solanacearum* including *fliC* gene and reclassified the bacterium as *R. pseudosolanacearum.* In the present study nucleotide sequence identity and phylogenetic tree (based on *fliC* gene) was constructed and it was found that the RSG01 and RSG03 clustered together in a sub group. That group again clustered with RGS02. *fliC* gene sequence of RSG02 isolate was very much close to USA isolates DQ657703 and DQ657701 of GenBank. *fliC* gene sequence of RSG01 and of RSG03 isolate were very much close to Indian isolate KF920693 and Japanese isolate KF275630 as recorded in GenBank. From the phylogenetic tree of *fliC* gene, RSG01 and RSG03 were very much close to each other (98-99% similarity). 94% similarity was found when the cluster was compared with RSG02 isolate.

Control of bacterial wilt by several antagonistic bacteria have been done in the past (Anuratha and Gnanamanickom, 1990; Tan et al 2013; Vanitha et al. 2010;

In the present study three antagonistic bacteria were isolated from soil. Those antagonistic bacteria were coded as HS01, HS02 and HS03. Among the three bacteria isolate HS01 showed best antagonism and restricted growth of *Ralstonia solanacearum* (isolate RSG01) significantly.

All the three antagonistic bacteria were studied for their morphological and biochemical characteristics. From the study, it was found the all the bacteria were Gram positive and rod shaped. From the biochemical tests it was also found that the bacteria were very much like *Bacillus* sp. Out of the three antagonistic bacteria, the most antagonistic bacteria were selected for molecular identification by PCR based method. After sequence identity and phyllogenetic tree construction the organism (isolate HS01) was identified as *Bacillus cereus.* The sequence of the 16S ribosomal RNA gene (partial) was submitted to GenBank and accession no. (KC959841) of the sequence was procured from GenBank.

After the characterization of the antagonistic bacteria they were considered to be tested for disease management in whole plants in pots and also in fields. *Bacillus cereus* isolate HS01 was best among the tested antagonists when applied separately. It could check wilt disease up to 20 days in pots. In field condition only *Bacillus cereus* (isolate HS01) could check the disease significantly.

Trichoderma harzianum, a well known biocontrol agent was also procured and was tested against *R. solanacearum* both by *in vitro* (Dual culture technique) as well as *in vivo* (by application in whole plant). *T. harzianum* could check the growth of *R. solanacearum* completely when tested *in vitro*. *T. harzianum* could reduce the disease (wilting index) up to 80% in sterilized soil in potted plants.

Some plants possess antibacterial activity (Nascimento et al., 2000) In the present study eight plant extracts have been tested for their potentiality to

check the growth of *R. solanacearum.* Plant extracts of *Zingiber officinale, Azadirachta indica* and *Camellia sinensis* could inhibit the growth of the *R. solanacearum* significantly. From the results of *in vivo* studies it was evident that *Camellia sinensis* leaf extract could successfully control *R. solanacearum* caused wilt in tomato plants.

Induction of resistance by chemicals is an alternative eco-friendly approach of plant defense. This is being followed in a number of crops against a variety of pathogens. Conrath et al., (2015) has described it as priming or potentiation or sensitization of plants to enhance inherent capacity of defense by some chemical inducers. Some known chemical inducers *viz.* β-aminobutyric acid (BABA), Salicylic acid (SA) and Abscisic acid (ABA) has been reported to induce resistance against a wide range of pathogens in a number of plant species. (Cohen et al., 1994; Chamsai et al., 2004).

Many plant enzymes are associated with ISR of plants. These enzymes may be classified on the basis of their major activities such as oxidative enzymes [peroxidase (PO) and polyphenol oxidase (PPO)] which catalyze the formation of lignin and other oxidative phenols. PO and PPO take part in the formation of defense barriers for the pathogen to the plant structure (Avdiushko et al., 1993). Other two enzymes tyrosine anmmonia lyase (TAL) and phenylalanine ammonia lyase (PAL) are related to phytoalexin and phenolic compound biosynthesis (Bashan et al., 1985; Beaudoin-Eagan et al., 1985). Chitinase and β-1,3-glucanase are hydrolyzing enzymes and have been reported to be active in defense against fungal pathogens. Some other enzymes such as super oxide dismutase, catalase, lipoxygenase, ascorbate peroxidase (APX) and proteinase inhibitors have also been correlated with defense in plants (Annapurna et al. 2007). Considering the importance of induction of defense in tomato plants against *Ralstonia solanacearum* (isolate RSG01), three different defense related enzymes (PAL, PPO, PO) were assessed to know their increased activity, if any, following application of three different chemical inducers (BABA, SA and ABA).

In conclusion, the present study reveals several new findings. Isolation and identification of 26 pathogenic *Ralstonia solanacearum* isolates were done from tomato plants of North Bengal. Three different antagonistic bacteria (*Bacillus cereus*) have been isolated and identified. *Trichoderma harzianum* found to be potential in controlling the disease caused by *R. solanacearum*. One of the botanical (*Camellia sinensis* leaf extract) found to be effective in controlling the disease. Alternatively SA and BABA also could reduce the disease following induction of defense in tomato plants.

Chapter-6

BIBLIOGRAPHY

Abdurahman, A., Parker, M. L., Kreuze, J., Elphinstone, J. G, Struik, P.C., Kigundu, A , Arengo, E and Sharma , K (2019) Molecular Epidemiology of *Ralstonia solanacearum* Species Complex Strains Causing Bacterial Wilt of Potato in Uganda . *Phytopathology* **109**:1922-1931

Acero J L, Benitez F J, Real F J and Gonzalez M. (2008) Chlorination of organophosphorus pesticides in natural waters. *J Hazard Mater.* **153:** 320–328.

Ajilogba, C. F. and Babalola, O. (2013) Integrated management strategies for tomato Fusarium wilt. *Biocontrol-Science* **18**(3):117-27

Akhtar, M. and A. Malik. (2000) Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: A review. *Bioresourse Technology* **74:**35–47.

Altschul,S.F. (1997) In Suhai,S. (ed.), Theoretical and Computational Methods in Genome Research. Plenum Press, New York, pp. 1–14.

Alvarez, B., Vasse, J., Le-Courtois, V., Trigalet-De´mery, D., Lo´pez, M. M., Trigalet, A., (2008) Comparative behavior of *Ralstonia solanacearum* biovar 2 in diverse plant species. *Phytopathology* **98**: 59–68.

Alyie, N., C. Fininsa and Hikias. Y. (2008) Evaluation of rhizosphere bacterial antagonists for their potential to bio-protect potato (*Solanum tubersoum*) against bacterial wilt (*Ralstonia solanacearum*). *Biological Control* **47:** 282-288.

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H., Oyaizu, H.(2000) Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* **50**: 1563-1589

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A. and Boucher, C. A. (1992) Transcriptional organization and expression of the large hrp

gene cluster of *Pseudomonas solanacearum* . *Molecular Plant-Microbe Interactions* 5: 187–193.

Artal, R.B., Gopalakrishnan C., Thippeswamy B. (2012).An efficient inoculation method to screen tomato, brinjal and chilli entries for bacterial wilt resistance. *Pest Management in Horticultural Ecosystems*., **18** (1): 70- 73.

Aslam,M.N., Mukhtar,T., Ashfaq,M. & Hussain,M.A., (2017) Evaluation of chili germplasm for resistance to bacterial wilt caused by *Ralstonia solanacearum.Australasian plant pathology* **46**, 289–292

Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A.; Struhl, K. (1992) Current protocols in molecular biology. New York: Greene Publishing Association; Wiley-Interscience,. v.1.

Bailey., K L and Lazarovits, G.(2003) Suppressing soil-borne diseases with residue management and organic amendments. *Soiland Tillage Research.* **72:**169–180.

Barrow, G.H. and Feltham, R.K.A. (1993) Cowan and Steel's Manual for Identification of Medical Bacteria. 3rd Edition, Cambridge University Press, Cambridge, 331.

Bartholomew, J. W. (1962) Variables Influencing Results, and the Precise Definition of Steps in Gram Staining as a Means of Standardizing the Results Obtained. Stain Technology **37:** 139-155.

Beckers, G. J. and Conrath, U. (2007). Current Opinion in Plant Biology 10: 425–431

Bekele, K. and Berga, L. (2001) Effects of preceding crop, variety and post emergence cultivation (hilling) on the incidence of bacterial wilt. PP 516-523 in Root Crops in the 21st Century. Proceedings of the Seventh Triennial Symposium of the International Society for Tropical Root

Crops-Africa Branch (ISTRC-AB).International Conference Center.pp. 11-17.

Bergsma-Vlami, M., van de Bilt, J. L. J., Tjou-Tam-Sin, N. N. A., Westenberg, M., Meekes, E. T. M., Teunissen, H. A. S. and Van Vaerenbergh J. (2018) Phylogenetic assignment of *Ralstonia pseudosolanacearum* (*Ralstonia solanacearum* Phylotype I) isolated from *Rosa* spp. *Plant Disease* **102:** 2258-2267.

Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R. and Schuch, W. (1989) The EMBO journal 8: 1899-1906.

Bias, H. P. (2004) How plant communicate using the underground information super highway. *Trends in Plant Science* **9:** 26-32.

Boshou, L. (2005) A broad review and perspective on breeding for resistance to bacterial wilt. In: Allen C, Prior P, Hayward AC, editors. *Bacterial Wilt Disease and the Ralstoniasolanacearum Species Complex.* American Phytopathological Society Press; St Paul, MN: pp. 225–238.

Boudazin G, Le Roux AC, Josi K, Labarre P and Jouan B (1999) Design of division specific primers of Ralstonia solanacearum and application to the identification of European isolates. European Journal of Plant Pathology 105: 373–380

Brotman Y, Normantovich M, Goldenberg Z, Zvirin Z, Kovalski I, Stovbun N, Doniger T, Bolger AM, Troadec C, Bendahmane A, Cohen R, Katzir N, Pitrat M, Dogimont C, Perl-Treves R. (2013) Mol Plant 6(1):235–8.

Buddenhagen, I. (1986) Bacterial wilt revisited. Pages 126-143 in: Bacterial Wilt Disease in Asia and the South Pacific, G. J. Persley, ed. ACIAR, Canberra, ACT, Australia.

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

Bufe, A., Spangfort, M. D., Kahlert, H., Schlaak, M. and Becker, W. M. (1996) Planta 199: 413-415.

Canale F., A. Peralta, and M. Colombo. 1983. Comparison of serological techniques for the detection of P. solanacearum. *Fitopatologia* **18**: 48-52

Caruso, P., Palomo, J. L., Bertolini, E., A ´lvarez, B., Lo ´pez, M. M. & Biosca, E. G. (2005). Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: recovery of stressed cells at low temperatures. *Appl Environ Microbiol* **71**, 140–148

Chaikam, V. and Karlson, D. T. (2010). Comparison of structure, function and regulation of plant cold shock domain proteins to bacterial and animal cold shock domain proteins. *BMB Reports* **43:** 1–8.

Chamsai, J., Siegrist, J., and Buchenauer, H. (2004) Mode of action of the resistance-inducing 3-aminobutyric acid in tomato roots against *Fusarium* wilt. *Journal of Plant Diseases and Protection* **111:** 273–291.

Chaudhry, Z. and Rashid, H. (2011) Isolation and characterization of *Ralstonia solanacearum* from infected tomato plants of SoanSkesar valley of Punjab. *Pakistan Journal of Botany* **43:** 2979-2985.

Choudhary, D. K., Prakash, A. and Johri, B. N. (2007) Indian Journal of Microbiology 47: 289-297.

Choudhary,D.K., Nabi, S. UN., Dar, M.S. and Khan, K.A.(2018) *Ralstonia solanacearum*: A wide spread and global bacterial plant wilt pathogen. *Journal of Pharmacognosy and Phytochemistry* **7**(2): 85-90

Christensen, A. B., Cho, B. H., Næsby, M., Gregersen, P. L., Brandt, J., Madriz-Ordeñana, K., Collinge, D. B. and Thordal-Christensen, H. (2002) Molecular Plant Pathology 3:135–144.

Christopoulou M, Wo SR, Kozik A, McHale LK, Truco MJ, Wroblewski T, Michelmore RW. (2015) G3 (Bethesda) 5(12):2655–69.

Ciampi-Panno, L., Fernandez, C., Bustamante, P., Andrade, N., Ojeda, S. and Conteras, A. (1989) Biological control of bacterial wilt of potatoes caused by *Pseudomonas solanacearum. American potato journal* **66:** 315-332.

Cindy, E., Morris, C. E., and Moury, B. (2019) Revisiting the Concept of Host Range of Plant Pathogens. *Annual Review of Phytopathology* **57:** 63–90 .

Clark, C. A. and Moyer, J. W. (1988) Compendium of Sweet Potato Diseases. APS Press, American Phytopathological Society, St. Paul, MN, USA, pp. 74.

Cohen, Y., Niderman, T., Mösinger, E. and Fluhr, R. (1994) β-Aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. *Plant Physiology* **104:** 59–66.

Converse, R.H. and Martin,R.R., (1990) ELISA methods for plant viruses. In R. Hampton, E. Ball, and S. de Boer (Eds.). Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. A Laboratory Manual. APS Press, St. Paul, Minnesota 179-196.

Coutinho, T.A., Roux, J., Riedel, K-H, Terblanche,J. and Wingfield,J.M. First report of bacterial wilt caused by *Ralstonia solanacearumon* eucalypts in South Africa *For. Path*. **30** : 205-210

Cowan, S.T and Steel, K.J (1993) Cowan and Steel's Manual for Identification of Medical Bacteria. 3rd Edition, Cambridge University Press, 199-241.

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

Dahal, D., Pich, A., Braun, H. P. and Wydra, K. (2010) Analysis of cell wall proteins regulated in stem of susceptible and resistant tomato species after inoculation with Ralstoniasolanacearum: a proteomic approach. *Plant Molecular Biology* **73:** 643–658. PMID: 20496099.

Dannon IA, Wydra K. Interaction between silicon amendment, bacterial wilt development and phenotype of *Ralstonia solanacearum* in tomato genotypes. *PhysiolMol Plant Pathol.* 2004;**64:**233–243.

Dasgupta S, Meisner C, Wheeler D, Xuyen K, Lam NT. Pesticide poisoning of farm workers-implications of blood test results from Vietnam. *Int J Hyg Environ Health.* 2007; **210:**121–132.

Denny, T. P. and Hayward, A. C. (2001) "Gram-Negative Bacteria *Ralstonia*," in: N. W. Schaad, J. B. Jones and W. Chun, Eds., Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd Edition, APS Press, St. Paul. pp. 151-174.

Denny, T.P.(2006) Plant Pathogenic Ralstonia species. p. In: Gnanamanickam SS (Ed.). Plant Associated Bacteria, Springer, The Netherlands. pp. 573-644

Dewen, Q., Yijie, D., Yi, Z., Shupeng, L. and Fachao, S. (2017) Molecular Plant-Microbe Interactions 30: 355-360.

Dhingra, O.B. and Sinclair, J.B. (1995) Basic Plant Pathology Methods. 2nd Edition, CRC Press, Boca Raton.

Dogimont C, Chovelon V, Pauquet J, Boualem A, Bendahmane A. (2014) Plant J 80(6):993–1004.

Dracatos P. M, Cogan N. O. I, Sawbridge T. I, Gendall A. R, Smith K. F, and Spangenberg G. C. (2009) BMC Plant Biology, 9: 62.

DrissaSilue, E. Pajot, Yigal Cohen (2002) Induction of resistance to downy mildew (Peronosporaparasitica) in cauliflower by DL-β-amino-n-butanoic acid (BABA). Plant Pathology 51(1):97 – 102 DOI:10.1046/j.1365-3059.2002.00649.x

Dutta, P. and Rahman, B. (2012) Varietal screening of tomato against bacterial wilt disease under subtropical humid climate of Tripura. *Journal of Farm Sciences* **2:** 40-43.

Echandi, E. (1991) Bacterial wilt. in: Compendium of Tobacco Diseases. H. D. Shew and G. B. Lucas, eds. *American Phytopathological Society*, St. Paul, MN,33-35

Edwards-Jones, G. (2008) Dobenefis accrue to 'pest control' or 'pesticides?': a comment on Cooper and Dobson. *Crop Protection* **27:** 965–967.

Ellis J., and Jones D. (1998) Current Opinion in Plant Biology 1: 288–293.

Elphinstone, J. G. (2005) "The current bacterial wilt situation: a global overview," in *Bacterial wilt Disease and the Ralstonia solanacearum Species Complex*, eds C. Allen, P. Prior, and A. C. Hayward, (St. Paul, MN: APS Press) pp. 9-28.

Elphinstone, J.G., Hennessy, J., Wilson, J. and Stead, D.E. (1996) Sensitivity of different methods for the detection of *Pseudomonas solanacearum* (Smith) Smith in potato tuber extracts. *Eur Mediterr Plant Prot Organ* **26**, 663–678.

Elphinstone, M. (2005) "The current bacterial wilt situation: a global overview," in *Bacterial Wilt Disease and the Ralstonia Solanacearum Species*

Complex, eds C. Allen, P. Piror, and A. C. Hayward (St. Paul, MN: APS Press) pp. 9-28.

Elphmstone, J. G., Hennessy, J., Wilson, J. K. and Stead, D. E. (1996) Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *Bulletin OEPP/EPPO Bulletin* **26:** 663-678.

Eshwar, A. K., Guldimann, C., Oevermann, A. and Tasara, T. (2017) Cold-Shock Domain Family Proteins (Csps) Are Involved in Regulation of Virulence, Cellular Aggregation, and Flagella-Based Motility in *Listeria monocytogenes*. *Frontiers in Cellular and Infection Microbiology* **7:** 453. PMID: 29124040 PMCID: PMC5662587.

Esther Amzalek ,Yigal Cohen (2007) Comparative Efficacy of Systemic Acquired Resistance-Inducing Compounds Against Rust Infection in Sunflower Plants. Phytopathology 97(2):179-86 DOI:10.1094/PHYTO-97-2-0179

Farag, N.S., F.G. Fawzi, S.I.A. El-Said and M.S. Mikhail.1986. Streptomycin in relation to potato brown rot control. Acta Phytopathologica Entomologica Hungarica **21:** 115-122

Fegan, M., and Prior, P. (2005) "How complex is the *Ralstonia solanacearum* species complex," in Bacterial wilt disease and the *Ralstonia solanacearum* species complex. ed. C. Allen, P. Prior and A. C. Hayward (St. Paul, MN: APS) pp. 449-462.

Fortnum, B. A. and Martin, S. B. (1998) Disease management strategies for control of bacterial wilt of tobacco in the southeastern USA, In P. Prior, C. Allen, and J. Elphinsone (ed.), Bacterial Wilt Disease: Molecular and Ecological Aspects. Springer. Heidelberg, New York. pp. 394–402.

Frías, M., Brito, N. and González, C. (2013) Molecular plant pathology 14:191-196.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G. Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J. P., Kessmann, H. and Ryals, J. (1996) The Plant Journal 10: 61-70.

Fu, Z. Q. and Dong, X. (2013) Annual review of plant biology 64: 839-863.

Gadeva, P. and Dimitrov, B.(2008).Genotoxic effects of the pesticides Rubigan, Omite and Rovral in root-meristem cells of *Crepiscapillaris* L. *Mutat Res.*;**652:**191–197.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S. and Ryals, J. (1993) Science-New York Then Washington 261: 754-754.

Gao, Q. M., Yu, K., Xia, Y., Shine, M. B., Wang, C., Navarre, D. andmKachroo, P. (2014) Cell reports 9: 1681-1691.

Garcıa, R.O.,Kerns,J.P and Thiessen ,L *Ralstonia solanacearum* Species Complex: A Quick Diagnostic Guide Plant Health Progress **20**:7–13

Garcia-Mas J, Benjak A, Sanseverino W, Bourgeois M, Mir G, González VM, Hénaff E, Câmara F, Cozzuto L, Lowy E, Alioto T, Capella-Guitérrez S, Blanca J, Cañizares J, Ziarsolo P, Gonzalez-Ibeas D, Rodríguez-Moreno L, Droege M, Du L, Alvarez-Tejado M, Lorente-Galdós B, Melé M, et al. (2012) PNAS 109(29):11872–7.

Genin , S and Boucher,C. (2002) *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome . *Molecular plant pathology* **3**(3), 111–118

Genin, S and Boucher,C .(2004) .Lessons learned from the genome analysis of *Ralstonia solanacearum. Annu. Rev. Phytopathol.* **42:**107–134

Genin, S. (2010) Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum* . *New Phytologist* **187:** 920-928.

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

Genin, S., Brito, B. Denny, T. and Boucher, C. A. (2005) Control of the Ralstoniasolanacearum Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Letters* **579:** 2077-81.

Getachew, A. H. (2009) Occurrence, Pathogen characterization and Evaluation of disease management options for tomato bacterial wilt (*Ralstonia solanacearum*) in major tomato growing areas of Ethiopia. pp. 201.

Getachew, A., Chemeda, F., Seid, A., and Wydra, K.(2011). Effects of soil Amendment on bacterial wilt caused by *Ralstonia solanacerum* and tomato yields in Ethiopia, *Journal of Plant Protection Research* **51 (1):** 72-76,

Gharaei F., Zamharir M.G (2017) Study of new NBS-LRR genes analogues in cucurbits native types

Ghosh, R. and Purkayastha, R. P. (2003) Current Science 85: 1782-1787

Goellner, K. and Conrath, U. (2008) European Journal of Plant Pathology 121: 233-242.

González VM, Aventín N, Centeno E, Puigdomènech P. (2014) Interspecific and intraspecific gene variability in a 1-Mb region containing the highest density of NBS-LRR genes found in the melon genome. BMC Genomics volume 15, Article number: 1131.

González VM, Müller S, Baulcombe D, Puigdomenech P. (2015) Evolution of NBS-LRR gene copies among Dicot plants and its regulation by members of the miR482/2119 superfamily of miRNAs. Mol Plant 8:329–31.

Gopalakrishnan, C., Singh, T. H. and Artal, R. B., (2014) Evaluation of eggplant accessions for resistance to bacterial wilt caused by *Ralstonia solanacearum* (E.F. Smith) Yabuuchi et al. *J. Hortl. Sci.* **9**(2):202-205

Gordon ,T.H., (2017) *Fusarium oxysporum* and the Fusarium wilt syndrome. Annual Review of Phytopathology **55**:23-39

Graham, J. and Lloyd, A. B. (1978) An improved indicator plant method for the detection of *Pseudomonas solanacearum* race 3 in soil. *Plant Dis. Rep.* **62:** 35-37.

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

Grover, A., Azmi ,W., Khurana, S. M. P. and Chakrabarti, S. K. (2009) Multiple displacement amplification as a pre-polymerase chain reaction (pre-pcr) to detect ultra low population of *Ralstonia solanacearum* (smith 1896) Yabuchi et al. (1996) *Letters in Applied Microbiology* **49**(5):539-43

Guo YL, Fitz J, Schneeberger K, Ossowski S, Cao J, Weigel D. (2011) Genome-wide comparison of nucleotide-binding site-leucine-rich repeat-encoding genes in Arabidopsis. Plant Physiol 157(2):757–69.

Haiko, J. and Westerlund-Wikström, B. (2013) The role of the bacterial flagellum in adhesion and virulence. *Biology* **2:** 1242-67.

Hales, B. A., Morgan, J. A. W., Hart, C. A. and Winstanley, C. (1998) Variation in flagellin genes and proteins of *Burkholderia cepacia*. *Journal of Bacteriology* **180:** 1110-1118.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber of *Colletotrichum lagenarium*. *Physiol. Plant Pathol.,* **20:** 73-82.

Hassan,S., Inam-ul-haq,M., Naz ,F. ,Tahir, M.I. and Ali,Z.(2016) In vitro investigations on host specificity of *Ralstonia solanacearum* among solanaceous crops and its biological control in tomato. *Pak. J. Bot.*, **48**(3): 1279-1287

Hayward, A. C. (1991) Biology and Epidemiology of Bacterial Wilt Caused by *Pseudomonas Solanacearum*. *Annual Review of Phytopathology* **29:** 65-87.

Hayward, A. C. (1994). The hosts of *Pseudomonas solanacearum*. In Bacterial Wilt: the Disease and its Causative Agent, Pseudomonas solanacearum, pp. 9–24. Edited by A. C. Hayward & G. L. Hartman. Wallingford: CAB International.

Hayward, A. C., El-Nashaar, H. M., Nydegger, U. and De Lindo, L. (1990) Variation in nitrate metabolism in biovars of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology* **69:** 269-280.

Hayward, A.C. (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum. Annu Rev Phytopathol* **29**, 65–87.

Hayward, A.C. (1994) The Hosts of *Pseudomonas solanacearum.* In: Hayward, A.C. and Hartman, G.L. (Eds.), Bacterial Wilt: The Disease and Its Causative Agent, Pseudomonas solanacearum, CAB International, Wallingford, pp. 9-24.

Hayward, A.C. 2000. *Ralstonia solanacearum*. In: Lederbergerg J. (Ed.) Encyclopedia of Microbiology Vol.4 San Diego, California. Academic Press. pp.32-42.

He, Y., Xu, T., Fossheim, L. E. and Zhang, X. H. (2012) FliC, a flagellin protein, is essential for the growth and virulence of fish pathogen. *PLoS One* **7:** e45070 PMID: 23024793 PMCID: PMC3443227.

Heim, S., Lleo´, M. D. M., Bonato, B.,Guzma´n, C. A. and Canepari, P. (2002) The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *Journal of Bacteriology* **184:** 6739-6745

Higa, A., Hidaka, T., Minai, Y., Matsuoka, Y. and Haga, M. (2001) Active oxygen radicals induce peroxidase activity in rice blade tissues. Biosci Biotechnol Biochem 65: 1852–1855. Jones, J. D. G. and Dangl, J. L. (2006) The plant immune system. Nature 444: 323-329.

Hoque, Md.E. and Mansfield, J. W. (2005) A simple and reliable method for pathogenicity tests of bacterial blight disease of rice. Bangladesh Journal of Botany. **34:** 11-16.

Huang S, Li R, Zhang Z, Li L, Gu X, Fan W, Lucas WJ, Wang X, Xie B, Ni P, Ren Y, et al. (2009) The genome of the cucumber, Cucumis sativus L. Nat Genet 41:1275–81

Huang, J., Carney, B. F., Denny, T. P., Weissinger, A. K. & Schell, M. A. (1995). A complex network regulates expression of eps and other virulence genes of Pseudomonas solanacearum. J Bacteriol 177: 1259-1267.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y. H., Yu, J. Q. and Chen, Z. (2010) Functional analysis of the Arabidopsis pal gene family in plant growth, development, and response to environmental stress. Plant Physiology 153: 1526–1538

Huynh, T. V., Dahlbeck, D. and Staskawicz, B. J. (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245:** 1374-1377.

InessaArif, Maria Batool, Peer M. Schenk, (2020) Plant Microbiome Engineering: Expected Benefits for Improved Crop Growth and Resilience, Trends in Biotechnology, **38:** 1385-1396.

J.F., Sambrook & Russell, D. (2001). Molecular Cloning: A Laboratory Manual (3-Volume Set).

Janse, J. D. (1988) A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. *Bulletin OEPP/EPPO Bulletin* **118:** 343-351.

Janse, J. D. (1996) Potato brown rot in western Europe–History, present occurrence and some remarks on possible origin, epidemiology and control strategies. *EPPO Bull* **26**:679-695

Janse, J. D., Beld, H. E. d., Elphinstone, J., Simpkins, S., Tjou-Tam-Sin, N. A. A., and Vaerenbergh, J. V.(2004) Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in Pelargonium zonale cuttings. *J. Plant Pathol.* **86**:147-155.

Janvier, C., Villeneuve, F. Alabouvette, C. EdelHermann, V. Mateille, T. and Steinberg. C. (2007) Soil health through soil disease suppression: Which strategy from descriptors to indicators? *Soil Biology and Biochemistry* **39:** 1-23.

JayeshPawaskar, J., Joshi, M. S., Navathe, S. and Agale, R. C. (2014) Physiological and Biochemical Characters of *Ralstonia solanacearum*. *International Journal of Research in Agricultural Sciences* **1:** 2348-3997.

Jia X, Yuan Y, Zhang Y, Yang S, Zhang X. (2015) Extreme expansion of NBS-encoding genes in Rosaceae. BMC Genet 16:48.

Jordá, L. and Vera, P. (2000) Local and systemic induction of two defenserelated subtilisin-like protease promoters in transgenic Arabidopsisplants. Luciferin induction of PR gene expression. Plant Physiology 124: 1049-1058.

Jupe F, Pritchard L, Etherington GJ, Mackenzie K, Cock PJ, Wright F, Sharma SK, Bolser D, Bryan GJ, Jones JD, Hein I. (2012) Identification and localisation of the NB-LRR gene family within the potato genome. BMC Genomics 13:75

Katafiire, M., Adipala, E., Lemaga, B., Olanya, M., El-Bedewy, R. and Ewell, P. (2005) Management of bacterial wilt of potato using one-season rotation crops in southwestern Uganda. Bacterial Wilt Disease and the Ralstoniasolanacearum Species Complex. American Phytopathological Society Press, St. Paul, MN, pp.197-204.

Kaur, A. and Kolte, S. J. (2001) Protection of mustard plants against staghead phase of white rust by Foliave treatment with benzothiadiazole. An activator of plant defense system. J. Mycol. Pl. Pathol. 31: 133-138.

Kawamura, Jun and Takeo, Tadakaju. (1989) Antibacterial Activity of Tea Catechin to Streptococcus mutans. Volume **36** Issue 6 Pages 463-467

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

Kelman, A. (1956) Factors influencing viability and variation in cultures of Pseudomonas solanacearum. *Phytopathology* **46:** 16–17.

Keto-Timonen, R., Hietala, N., Palonen, E., Hakakorpi, A., Lindström, M. and Korkeala, H. (2016) Cold Shock Proteins: A Minireview with Special Emphasis on Csp-family of Enteropathogenic Yersinia. *Frontiers in Microbiology* doi: 10.3389/fmicb.2016.01151.

Khetmalas, M. B. (1984) Studies on wilt of groundnut (*Arachis hypogaea* L.) caused by *Pseudomonas solanacearum* M. Sc. (Agri.) thesis submitted to Konkan Krishi Vidyapeeth, Dapoli, India.

Kim, D. S. and Hwang, B. K. (2014) An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. Journal of

Kinyua, Z. M., Smith, J. J., Lung'aho, C., Olanya, M. and Priou, S. (2001) On-farm success and challenges of producing bacterial wilt free tubers in seed plots in Kenya. *African Crop Science Journal* **9:** 279-285.

Kirkegaard JA, Wong PTW, Desmarchelier JM. *In vitro* suppression of fungal root pathogens of cereals by *Brassica* tissues. *Plant Pathol.* 1996; **45:**593–603

Knapp, S., L. Bohs, M. Nee and D.M. Spooner. 2004. Solanaceae-A model for linking genomics with biodiversity. Comparative and Functional Genomics **5**: 285-291

Kubota, R,. Vine, B. G., Alvarez, A. M., and Jenkins, D. M. (2008) Detection of *Ralstonia solanacearum* by Loop-Mediated Isothermal Amplification. Bacteriology Open Access. Kubota, R., Vine, B. G., Alvarez, A. M., & Jenkins, D. M. (2008). Detection of Ralstonia solanacearum by Loop-Mediated Isothermal Amplification. *Phytopathology* **98:** 1045-1051.

Kuć, J. (1982) Induced immunity to plant disease. Bioscience 32: 854–860.

Kucharek, T. (1998) Bacterial wilt of row crops in Florida. University of Florida, Cooperative Extension Service, Institute of Food and Agricultural Sciences, Florida, U. S. Cir. 1207. p. 9.

Kurabachew H, Wydra K. Induction of systemic resistance and defense-related enzymes after elicitation of resistance by rhizobacteria and silicon application against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*) *Crop Prot.* 2014;**57:**1–7.

Kurabachew, H and Ayana, G (2016) Bacterial wilt caused by ralstonia solanacearum in ethiopia: status and management approaches: A REVIEW *Int. J. Phytopathol.* 05 (03) 107-119

Kurabachew, H. and Ayana G., (2017) Bacterial wilt caused by *Ralstonia solanacearum* in Ethiopia: status and management approach: a review. *International Journal of Phytopathology* **5:** 107-119.

Kurabachew, H. and Wydra, K. (2013) Characterization of plant growth promoting rhizobacteria and their potential as bio-protectant against tomato bacterial wilt caused by *Ralstonia solanacearum*. *Biological Control* **67:** 75-83.

Kurabachew, H., Fasil A. and Yaynu, H. (2007) Evaluation of Ethiopian isolates of *Pseudomonas fluorescens* as biocontrol agent against Potato bacterial wilt caused By *Ralstonia* (*Pseudomonas*) *solanacearum*. *Acta Agriculturae Slovenica* **90:** 125-135.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995) Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. MPMI-Molecular Plant Microbe Interactions 8: 863-870.

Lemaga, B., Kakuhenzine, R.,Kassa ,B., Ewell ,P.T and Priou,S.(2005). Integrated control of potato bacterial wilt in eastern Africa: the experience of African highlands initiative, p. 145–158. In Allen,C., Prior ,P., and Hayward , A.C (ed.), Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. *American Phytopathological Society Press*.

Lemaga, B., Siriri, D., and Ebanyat, P. (2001). Effect of soil amendments on Bacterial Wilt incidence and yield of potatoes in Southwestern Uganda. *African Crop Science Journal* **9 (1):** 267-278.

Lemessa, F. and Zeller, W. (2007) Isolation and characterisation of *Ralstonia solanacearum* strains from Solanaceae crops in Ethiopia. *Journal of Basic Microbiology* **47:** 40-9.

Leppla, N., Momol, T., Nesheim, N. & Dusky, J. (2004) Plant, animal and human protection, FAS 2 Focus Area. University of Florida / IFAS.

Li, J., Hansen, B. G., Ober, J. A., Kliebenstein, D. J. and Halkier, B. A. (2008) Subclade of flavin-monooxygenases involved in aliphatic glucosinolate biosynthesis. Plant Physiology 3:1721-1733.

Liu, Y., Orsi, R. H., Gaballa, A., Wiedmann, M., Boor, K. J. and Guariglia-Oropeza, V. (2019) Systematic review of the *Listeria monocytogenes* σ^B regulon supports a role in stress response, virulence and metabolism. *Future Microbiology* **14:** 801–828.

M. T. Momol, S. M. Olson, Jeff Jones (2003) Effects of Plant Essential Oils on *Ralstonia solanacearum* Population Density and Bacterial Wilt Incidence in Tomato. Plant Disease **87(4):**423-427 DOI:10.1094/PDIS.2003.87.4.423

Machmud, M. and Suryadi,Y. (2008) Detection and identification of *Ralstonia solanacearum* strains using the indirect elisa technique. Indonesian Journal of Agriculture **1**(1), 2008: 13-21

Machmud, M., Jumanto H., Roechan M., I. Manzila, Yadi S., dan M.A. Suhendar. 1998. Perbaikan Teknik Serologi dan Biomolekuler untuk Deteksi dan Identifikasi Patogen Tumbuhan serta Perakitan Perangkatnya Guna Menunjang Program Produksi Benih. Laporan Hasil Penelitian 1997/1998. Balai Penelitian Bioteknologi Tanaman Pangan, Bogor.

Machmud, M., Y. Suryadi, M.A. Suhendar, Jumanto H., dan Roechan M. 1999. Perakitan Perangkat ELISA untuk Deteksi dan Identifikasi Rs, SMV, PSG

dan Xcg dengan Antibodi Poliklonal. Laporan ROPP 1998-1999. UPT Perkebunan, Bogor.

Machmud, M., Yadi S., M.A. Suhendar, Jumanto H., I. Manzila, dan M. Roechan. 1997. Produksi perangkat ELISA untuk deteksi virus bilur kacang tanah, virus kerdil kedelai, dan bakteri layu (P. solanacearum). Laporan ROPP 1996/1997. Balai Penelitian Bioteknologi Tanaman Pangan, Bogor.

Malu, Samuel &Obochi, G &Tawo, E &Nyong, Bassey. (2009). Antibacterial activity and medicinal properties of Ginger (Zingiber officinale). Global Journal of Pure and Applied Sciences. Doi: 10.4314/gjpas.v15i3-4.48561.

Manoj Kumar, M., Srinivasa, V., and Kumari, M. (2018) Screening of Tomato Line/Varieties for Bacterial Wilt (*Ralstonia solanacearum*) Resistance in Hill Zone of Karnataka. *International Journal of Current Microbiology and Applied Sciences* **7:** 1451-1455.

Mansfield J, Genin S, Magori S et al., 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology* **13**, 614–29

Mauch-Mani, B. and Slusarenko, A. J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to Peronospora parasitica. The Plant Cell 8: 203-212.

McCarter, S. M., Dukes, P. D. and Jaworski, C. A. (1969) Vertical distribution of *Pseudomonas solanacearum* in several soils. *Phytopathology* **59:**1675-1677.

McCarter, S.M.(1991) Bacterial wilt. In: Jones JB, Jones JP, Stall RE, Zitter TA (Eds.) Compendium of tomato diseases. *American Phytopathological Society*. St. Paul, Minnesota, USA. pp.28-29.

McLaughlin, R.J. and T.A. Chen.(1990) ELISA methods for plant pathogenic prokaryotes. p. 197-201. In R. Hampton and S.H. De Boer (Eds.). Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. A Laboratory Manual. APS Press, St Paul, Minnesota

Meena, B., Marimuthu, T. and Velazhahan, R. (2001) Salicylic acid induces systemic resistance in groundnut against late leaf spot caused by Cercosporidium personatum. Journal of Mycology and Plant Pathology 31: 139-145.

Mepharishvili , G., Sikharulidz ,Z., Thwaites,R., Tsetskhladze,T., Dumbadze,R., Gabaidze, M.and Muradashvili,M.First confirmed report of bacterial wilt of tomato in Georgia caused by *Ralstonia solanacearum.New Disease Reports* **25**, 16

Metraux ,J .P., Ahl-Goy, P., Staub, T., Speich, T., Steinemann, A., Ryals , J. and Ward, E. (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In: Hennctcke, H. and Verma, D. P. S. (eds.) Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. Kluwer Academic Publishers, Dordrecht,The Netherlands, pp 432-439.

Michel, V. V. and Mew. T.W.(1998) Effect of soil amendment on the survival of *Ralstonia solanacearum* in different soils. *Phytopathology* **88:**300-305.

Mihaliak, C. A., Gershenzo, J. and Croteau, R. (1991) Lack of rapidmonoterpene turnover in rooted plants, implications for theories of plant chemical defense. *Oecologia* **87:** 373-376.

Mitchell, E., Houles, C., Sudakevitz, D., Wimmerova, M., Gautier, C., Perez, S., Wu, A. M., Gilboa-Garber, N. and Imberty, A. (2002) Structural basis for

oligosaccharide-mediated adhesion of Pseudomonas aeruginosa in the lung of cystic fibrosis patients. *Nature Structural & Molecular Biology* **9:** 918-921.

Momol, P. G. C., and Champoiseau, T. M. (2009)Bacterial wilt of tomato. https:// plantpath.ifas.ufl.edu/rsol/RalstoniaPublications_PDF/BacterialWiltTomato_ Revised_PDF.pdf.

Mondal, B., Bhattacharya, I. and Khatua, D.C. (2014) Incidence of bacterial wilt disease in West Bengal, India. *Academia Journal of Agricultural Research* **2:** 139-146.

Morata J & Puigdomènech P. (2017) Variability among Cucurbitaceae species (melon, cucumber and watermelon) in a genomic region containing a cluster of NBS-LRR genes. BMC Genomics volume 18, Article number: 138.

Mou, Z., Fan, W. and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113:935-944.

Muhire BM, Golden M, Murrell B, Lefeuvre P, Lett J, et al. (2014) Evidence of pervasive biologically functional secondary structures within the genomes of eukaryotic single-stranded DNA viruses. J Virol **88:** 1972–1989.

Muthoni, J., Somalis, H. and Melis, R. (2012)Management of Bacterial Wilt [Rhalstonia solanacearumYabuuchi et al., 1995] of Potatoes: Opportunity for Host Resistance in *Kenya Journal of Agricultural Science*.**4:** 64-78.

Nakaho, K., Hibino, H. and Miyagawa H. (2008) Possible Mechanisms Limiting Movement of Ralstoniasolanacearum in Resistant Tomato Tissues. *Journal of Phytopathology* **148:** 181 – 190.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y. and Yoshida, S. (2003) Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. The Plant Journal 33: 887-898.

Nascimento, G. G. F., Locatelli, J., Freitas, P. C., & Silva, G. L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology* Doi:10.1590/s1517-83822000000400003

Nesmith, W. C., and S. F. Jenkins, JR. (1979) A selective medium for the isolation and quantification of *Pseudomonas solanacearum* from soil. *Phytopathology* **69:** 182-185.

Norman DJ, Chen J, Yuen JMF, Mangravita-Novo A, Byrne D, Walsh L. Control of bacterial wilt of geranium with phosphorous acid. *Plant Dis.* 2006; **90:**798–802

Novitsky, J. A. & Morita, R. Y. (1976). Morphological characterization of small cells resulting from nutrient starvation of a pyschrophilic marine Vibrio. *Appl Environ Microbiol* **32**, 617–622.

Novitsky, J. A. and Morita, R. Y. (1976) Morphological characterization of small cells resulting from nutrient starvation of a pyschrophilic marine Vibrio. *Applied and Environmental Microbiology* **32:** 617-622.

OEPP/EPPO.2004. Ralstonia solanacearum. European and Mediterranean Plant Protection Organization Bulletin **34:** 173-178

Oliver, J. D. (2005) The viable but nonculturable state in bacteria. *Journal of Microbiology* **43:** 93-100.

Passardi, F., Longet, D., Penel, C. and Dunand, C. (2004) The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 65: 1879-1893

Paul, P. K., Sharma, P. D. (2002) Azadirachta indica leaf extract induces resistance in barley against leaf stripe disease. Physiol. Mol. Plant Pathol 61: 3-13.

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. and Bakker, P. A. (2014) Induced systemic resistance by beneficial microbes. Annual review of phytopathology 52: 347-375.

Popoola, A. R., Ganiyu, S. A., Enikuomehin, O. A., Bodunde, J. G., Adedibu, O. B., Durosomo, H. A. and Karunwi, O. A. (2015) *Nigerian Journal of Biotechnology* **29:** 1-10.

Pradhanang PM, Ji P, Momol MT, Olson SM. Application of acibenzolar-s-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. *Plant Dis.* 2005; **89:**989–993

Priou, S. and Aley, P. Chujoy, E., Lemaga B. and French E. (1999) Integrated Control of Bacterial Wilt of Potato. CIP slide Training Series IV.3. International Potato Center, Lima, Peru.

Qi Huang, Q., Yan, X. and Wang, J. F. (2012) Improved biovar test for *Ralstonia solanacearum. Journal of Microbiological Methods* **88:** 271-4. PMID: 22200550.

Rahman M. F., Islam, R., Rahman, T. and Meah, B. (2010) *Progressive Agriculture* **21:** 9-19.

Ramirez , M., Moncada, R.N., Villegas-Escobar, V., Jackson, R.W. and Ramirez, C.A. (2020) Phylogenetic and pathogenic variability of strains of Ralstonia solanacearum causing moko disease in Colombia *Plant Pathology* **69 :**360–369

Robinson-Smith, A., Jones, P., Elphinstone, J. G. and Forde, S. M. D. (1995) Production of antibodies to *Ralstonia solanacearum*, the causative agent of bacterial wilt. *Food and Agricultural Immunology* **7:** 67-79.

Rollins, D. M. and Colwell, R. R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology* **52:** 531–538.

Ross, A. F. (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology 14: 340–358.

Roszak, D. B. and Colwell, R. R. (1987) Survival strategies of bacteria in the natural environment. *Microbiol Rev* **51:** 365–379.

Rudrappa, K. B., Suryawanshi, A. P., Punitkumar N. D., Ganesh, J. K., Lambani, K. and Singh, R. (2016) Cultural and Biochemical Characterization of Ralstoniasolanacearum Causing Bacterial Wilt in Tomato. *Journal Of Pure And Applied Microbiology* **10:** 3111-3115.

Ruiz, J. A., Lo´ pez, N. I., Ferna´ndez, R. O. & Me´ ndez, B. S. (2001) Polyhydroxy alkanoate degradation is associated with nucleotide accumulation and enhances stress resistance and survival of *Pseudomonas oleovorans* in natural water microcosms. *Applied and Environmental Microbiology* **67:** 225–230.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y. (1996) Systemic acquired resistance. Plant Cell 8: 1809-1819.

S. Arivazhagan, L. Ganesan and T. G. Subash Kumar, "Texture classification using Curvelet Statistical and Co-occurrence Features," *18th International Conference on Pattern Recognition (ICPR'06)*, 2006, pp. 938-941.

Sadasivam, S. and Manickam, A. “Biochemical Methods,” New Age International (P) Limited, New Delhi, Vol. **2**. 1996, pp. 124-126.

Sah S, Sahgal M, Singh R (2017) Concomitant ability of siderophore against iron paucity and fusarium wilt in lycopersicon esculentum. Biosci Biotech Res Asia **14**:319–328

Saha S, Chant D, Welham J, McGrath J (2005) A Systematic Review of the Prevalence of Schizophrenia. PLoS Med **2:** e141.

Saile, E., McGarvey, J.A.,Schell, M.A.; Denny, T.P.(1997) Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by Ralstonia solanacearum. *Phytopathology* , **87**, 1264–1271.

Saitou N, Nei M. The neighbor-joining method: a new method for reconstruction phylogenetic trees. Mol Biol Evolu1997;4:406-25.

Saleim,M.A.A., Abo-Elyousar,K.A.M., Abd-El- Moneem , K.M. and Saead , F.A.,(2014) First Report of Bacterial Wilt caused by *Ralstonia solanacearum* Biovar 2 Race 1 on Tomato in Egypt .*Plant Pathol. J.* **30**(3):299-303

Sally, D. and Mathew, K. (2015) Evaluation of Endophytic Microbial Consortium for the Management of Bacterial Wilt of Tomato Caused by Ralstonia solanacearum. *Journal of Biological Control* **29:** 148.

Samaranayake *et al.* (2005) A conceptual framework for supply chain management: a structural integration", Supply Chain Management, **10:** 47-59.

Sanjula Sharma, BS Sohal (2017) Insight into the Biochemical Changes Induced in Brassica juncea (var. RLM619) on Application of Elicitors Alone and in Combinations. Indian Journal of Agricultural Biochemistry 30(2):121 DOI:10.5958/0974-4479.2017.00020.X

Santos, BM., Gilreath,JP., Motis,TN., Noling, JW., Jones, JP.,and Norton, JA. (2006) Comparing methyl bromide alternatives for soil-borne disease, nematode and weed management in fresh market tomato. *Crop Protection*; **25:** 690–695.

Schaad N. W. and Stall R. E. (1988) *Xanthomonas*. In: Schaad N.W. (ed.). Laboratory guide for Identification of Plant Pathogenic Bacteria, 2nd ed., APS Press, St. Paul, MN, USA. pp. 81-91.

Schaad, N. W. and Jones, Jeff & W, Chun. (2001). Laboratory Guide for Identification of Plant Pathogenic Bacteria.

Schell, M. A. (2000) Control of Virulence and Pathogenicity Genes of Ralstonia Solanacearum by an Elaborate Sensory Network. Annual Review of Phytopathology, **38:** 263–292.

Schönfeld, J., Heuer, H., van Elsas, J. D., and Smalla, K. (2003) Specific and Sensitive Detection of *Ralstonia solanacearum* in Soil on the Basis of PCR Amplification of fliCFragments. *Applied and Environmental Microbiology* **69:** 7248–7256. PMCID: PMC309886. PMID: 14660373

Schulze-Lefert, P., Panstruga, R., (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* **16:** 117-25.

Seal, S.E. and J.G. Elphinstone, (1994) Advances in Identification and Detection of *Pseudomonas solanacearum*. In: Bacterial with, the Disease and its Causative Agent *Pseudomonas solanacearum*, Hayward, A.C. and G.L. Hartman (Eds.). CAB International, UK., pp: 35-57.

Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., van den Elzen, P. J. M. and Cornelissen' B. J. C. (1993) Only Specific Tobacco (Nicotiana tabacum) Chitinases and β-1,3- Glucanases exhibit antifungal activity. Plant Physiol. 101: 857-863. van Loon, L. C., Rep, M.

and Pieterse, C. M. (2006) Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol 44: 135-162

Shao ZQ, Xue JY, Wu P, Zhang YM, Wu Y, Hang YY, Wang B, Chen JQ. (2016) Large-scale analyses of angiosperm nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes reveal three anciently diverged classes with distinct evolutionary patterns. Plant Physiol 170(4):2095–109.

Sharma, D. and Singh, Y. (2019) Characterization of *Ralstonia solanacearum* isolates using biochemical, cultural, molecular methods and pathogenicity tests. *Journal of Pharmacognosy and Phytochemistry* **8:** 2884-2889.

Sikdar B, Bhattacharya M, Mukherjee A, Banerjee A, Ghosh E, Ghosh B, and Roy S. C. (2010) Genetic diversity in important members of Cucurbitaceae using isozyme, RAPD and ISSR markers. Biologia Plantarum 54: 135–140.

Singh, B.; Chaudhary, J. L.; Rajora, N. K., 2005. Nutritive evaluation of soybean straw in sheep and goats. *Indian J. Anim. Nutr.* **22:** 67-69.

Spoel, S. H. and Dong, X. (2012) How do plants achieve immunity? Defence without specialized immune cells. Nature reviews. Immunology 12: 89.

Stanford, E. E. and Wolf, F. A. (1917) Studies on Bacterium solanacearum. *Phytopathology* **7:** 155-165.

Stevenson, W. R., Loria, R., Franc, G. D., and Weingartner, D. P. (2001) Compendium of Potato Diseases. *American Phytopathological Society*, St. Paul, MN

Stobbs, L.W. and D. Barker. 1985. Rapid sample analysis with a simplified ELISA. *Phytopathology* **75**: 492-495.

Swanson, J. K., Yao, J., Tans-Kersten, J., and Allen, C. (2005) Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. *Phytopathology* **95:** 136-143.

Tahat, M. M. and Sijam, K. (2010) *Ralstonia solanacearum*: The Bacterial Wilt Causal Agent. *Asian Journal of Plant Sciences* **9:** 385-393.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution*, ***30*****:** 2725–2729.

Tans-Kersten, J., Brown, D. and Allen C (2004) Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment. *Molecular Plant-Microbe Interactions* **17:** 686-95. PMID: 15195951.

Tans-Kersten, J., Huang, H. and Allen, C. (2001) *Ralstonia solanacearum* needs motility for invasive virulence on tomato. Journal of Bacteriology **183:** 3597-605.

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

Trigiano, R. and Gray, D. J. (2004).Plant development and biotechnology.

Tsiri, Dimitroula&Graikou, Konstantia&Pobłocka-Olech, Loretta &Krauze-Baranowska, Miroslawa& Spyropoulos, Caroline &Chinou, Ioanna. (2009). Chemosystematic Value of the Essential Oil Composition of Thuja species Cultivated in Poland—Antimicrobial Activity. Molecules (Basel, Switzerland). **14.** 4707-15.

Van der Wolf, J. and Perombelon, M. (1997) Potato brown rot in temperate regions – a review. http://www.spud.co.uk/external/PROF/RESEARCH/scri/brownro3.htm

Van Elsas, J. D., Kastelein, P., van Bekkum, P., van der Wolf, J. M., de Vries, P. M. & van Overbeek, L. S. (2000). Survival of Ralstonia solanacearum biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology* **90**, 1358–1366.

van Loon, L. C., Rep, M. and Pieterse, C. M. (2006) Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol 44: 135-162

Van Overbeek, L. S., Bergervoet, J. H. W., Jacobs, F. H. H. & Van Elsas, J. D. (2004) The low-temperature-induced viable-butnonculturable state affects the virulence of Ralstonia solanacearum biovar 2. *Phytopathology* **94**: 463–469.

Vasse , J.,Frey, P. and Trigalet , A. (1995) Microscopic studies of Intercellular Infection and Protoxylem invasion of Tomato Roots by Pseudomonas solanacearum. *Mpmi* **8** (2): 241-251

Vasudeo Z, Sonika B (2009). Antimicrobial activity of Tea (Camellia sinensis). Biomed. Pharmacol. J. **2(1):** 173-175.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E. and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. The Plant Cell 6: 959-965.

VnElsas, J.D., Kastelein,P.,DeVries,P.M.& vanOverbeek,L.S.(2001). Effects of ecological factors on the survival and physiology of Ralstonia solanacearum bv. 2 in irrigation water. *Can J Microbiol* **47**: 842–854.

Wakimoto, S., Utatsu, I., Matsuo, N. & Hayashi, N. (1982) Multiplication of Pseudomonas solanacearum in pure water. *Nippon Shokubutsu Byori Gakkaiho* **48**, 620–627.

Walters, D. R., Ratsep, J. and Havis N. D. (2013) Controlling crop diseases using induced resistance: challenges for the future. Journal of Experimental Botany 64: 1263–1280

Wan H, Yuan W, Bo K, Shen J, Pang X, Chen J(2013)Genome-wide analysis of NBS-encoding disease resistance genes in Cucumis sativusand phylogenetic study of NBS-encoding genes in Cucurbitaceae crops .BMC Genomics ,volume 14, Article number: 109 (2013)

Wan H. J., and Chen J. F. (2010) Characterization of NBS-LRR resistance gene analogs from a high resistance to downy mildew introgression line from Cucumis hystrix x C. sativus. Acta Horticulture, 871: 573–578

Wang, J. F. and Lin, C. H. (2005) Integrated management of tomato bacterial wilt. AVRDC-The world vegetable center; Taiwan.

Wang, J.F. and Lin, C.H.(2005) Colonizing capacity of *Ralstonia solanacearum* tomato strains differing in aggressiveness on tomato and weeds. In: Allen C, Prior P,.Hayward AC (Eds.) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. *The American Phytopathological Society,* St.Paul Minnesota, USA. Pp. 73-79.

Whipps, J. M. and Gerhardson, B. (2007) Biological pesticides for control of seed- and soil-borne plant pathogens, In J.D. VanElsas, J.D. Jansson, and J.T. Trevors (ed.), Modern Soil Microbiology 2nd Edition. CRC Press, FL pp. 479–501.

Whipps, J. (2001) Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52:** 487–511.

Wicker,E. Grassart, L., Coranson-Beaudu,R., Mian, D'L.,Guilbaud, C., Fegan,M.,and Prior,P. *Ralstonia solanacearum* Strains from Martinique (French West Indies) Exhibiting a New Pathogenic Potential. *Applied and Environmental Microbiology* **73** (21):6790–6801

Wolde, Tesfaye & H, Kuma &Dumga, Kassahun &YabekerBeza, Abebe. (2018). Anti-Bacterial Activity of Garlic Extract against Human Pathogenic Bacteria. Journal of Pharmacovigilance. Doi: 10.4172/2329-6887.

Wydra K, Dannon E. Silicon as inducer of resistance in tomato against *Ralstoniasolanacearum*. *Bulletin OILB/SROP.* 2006; **29:**91–96.

Wyszogrodzka A. J, Williams P. H, and Peterson C. E. (1987) Multiple-pathogen inoculation of cucumber (Cucumis sativus) seedlings. Plant Disease 71: 275–280.

Xiaoman, S. I., Lin, Y., Guobing, L., Yafei, T. and Zifu, H. (2017) Identification and Genetic Characterization of *Ralstonia solanacearum* Species Complex Isolates from *Cucurbita maxima* in China. *Plant Science* **8:** 1794. PMCID: PMC5651519. PMID: 29093727.

Xue ,H., Lozano-Durán, R.and Macho, A.P.(2020) Insights into the Root Invasion by the Plant Pathogenic Bacterium *Ralstonia solanacearum*. *Plants* **9**: 516;

Yadessa, G. B., Van Bruggen, A. H. C., and Ocho, F.I (2010). Effects of different soil amendments on bacterial wilt caused by *Ralstonia solanacearum* and on the yield of tomato. *Journal of Plant Pathology* **92 (2):** 439-450

Yadi S., M. Machmud, Rusmadi, and M.A. Suhendar, M.A. (1998)Detection of *P. solanacearum* from latently infected potato tubers using ELISA and PCR techniques. Jurnal Biologi Indonesia **II**(3): 142-149.

Yoshioka, K., Nakashita, H., Klessig, D. F. and Yamaguchi, I. (2001) The Plant Journal 25: 149-157

Yuliar, Y., Nion A. and Toyota, K. (2015) Recent Trends in Control Methods for Bacterial Wilt Diseases Caused by *Ralstonia solanacearum*. Minireview. *Microbes and Environment* **30:** 1-11.

Zhou T, Wang Y, Chen JQ, Araki H, Jing Z, Jiang K, Shen J, Tian D. (2004) Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. Mol Genet Genomics 271(4):402

7. A. Appendix-I

Chemicals and Reagents

In addition to the common laboratory reagents, following chemicals were used during the present study.

Chemicals	**Company**
2, 3, 5-tripheny tetrazolium chloride	SRL Pvt. Ltd., Mumbai, India
10x Taq DNA buffer B	Genie, Bangalore
6x loading dye	Genie, Bangalore
6x loading dye	Genie, Bangalore
Acetic acid	SRL Pvt. Ltd., Mumbai, India
Agarose	SRL Pvt. Ltd., Mumbai, India
Ampicilin	HiMedia, Mumbai, India
Calcium chloride	HiMedia, Mumbai, India
CTAB	CDH Pvt. Ltd., New Delhi, India
DEPC-treated water	Chromus Biotech, Bangalore
dNTP mix	Genie, Bangalore
Eco RI	Promega Corporation, USA
EDTA	SRL Pvt. Ltd., Mumbai, India
Ethydium bromide	SRL Pvt. Ltd., Mumbai, India
Ethyl alcohol	JHI Co. Ltd., China
Forward and Reverse Primer	Sigma, USA
Glycerol	SRL Pvt. Ltd., Mumbai, India
IPTG	Promega Corporation, USA
Isoamyl alcohol	E. Merck, Mumbai, India
Isopropanol	SRL Pvt. Ltd., Mumbai, India
JM 109	Promega Corporation, USA
$MgCl_2$	Genie, Bangalore

NaCl	E. Merck, Mumbai, India
Oligo $(dT)_{18}$ primer	Promega Corporation, USA
pGEM- T easy cloning kit	Promega Corporation, USA
pGEM-T vector	Promega Corporation, USA
RNase	Promega Corporation, USA
Sodium Sulphite	E. Merck, Mumbai, India
T_4 ligase	Promega Corporation, USA
Taq DNA polymerase	Genie, Bangalore
Tris	SRL Pvt. Ltd., Mumbai, India
X- Gal	Promega Corporation, USA
β-marcaptoethanol	SRL Pvt. Ltd., Mumbai, India

7. B. Appendix II

Buffers and Solutions

 # Extraction buffer for 2X CTAB DNA (required for per gram leaf tissue):

Tris (1M):	500µl (pH 8.0)
NaCl (5M):	1.4ml
EDTA (0.5M):	200µl (pH 8.0)
β-marcaptoethanol:	10µl
Sterile water:	2.89ml
CTAB:	100mg

 # TE buffer

Tris-HCl	10 mM
EDTA	1 mM
Final pH	8.0

 # TAE buffer:

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml
Distilled water (final volume made up to)	1000 ml
Final pH	8.0

To make 1X TAE buffer, 1 ml 50X stock buffer was diluted in 49 ml distilled water to make final volume 50 ml.

 # Ethidium bromide (0.1 %) stock solution (10 ml):

100 mg ethidium bromide was dissolved in 10 ml distilled water; Stored in dark bottle at 4˚C.

 # Ampicillin stock solution (100 mg/ml):

One gram of ampicillin was dissolved in 10 ml of sterile distilled water. Solution was filter sterilized using Whatman poly ethersulfone membrane (0.2 µm pore size), stored in aliquots at -20°C.

 # X-Gal stock solution (20 mg/ml)

200 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was dissolved in 10 ml of N, N-dimethylformamide. The solution was stored at -200C in a dark bottle.

 # IPTG stock solution (100 mM)

240 mg of IPTG (Isopropyl-β-Dthiogalactopyranoside) was dissolved in 10 ml of sterile distilled water. The solution was filter sterilized and stored in aliquots at 4˚C.

 # Luria-Bertani (LB) Agar

To make Luria-Bertani (LB) Agar 25.0 g of powder was mixed with distilled water. Then agar powder was added at the rate of 1 % i.e., 10 g and the final volume was made up to 1.0 liter with distilled water. Then it was autoclaved at 15 psi pressure at 121˚C for 15 minutes.

 # Luria-Bertani (LB) Broth, Miller

To make Luria-Bertani (LB) Broth 25.0 g of powder was mixed with distilled water to make the final volume of 1.0 liter. Then it was autoclaved at 15 psi pressure at 121˚C for 15 minutes.

 # LB + Ampicillin + X-gal + IPTG plates

Autoclaved LB agar medium was allowed to cool to 50-550C. Required volume of ampicillin stock solution was added to the medium to reach the final concentration of 100 µg/ml. The medium was gently mixed and poured on 90-mm size petri plates. The plates were allowed to solidify and dried open under laminar for 30 min. 40 µl of X-Gal stock solution (20mg/ml) and 40 µl of 100

mM IPTG stock solution were spread evenly over each plate with sterile glass spreaders.

7. C. Appendix-III

Growth media

 # POTATO DEXTROSE BROTH (PDB)

Peeled potato	:	40 g
Dextrose	:	2 g
Distilled water	:	100 ml

Potato was peeled and cut into small square pieces. There after 40 gram of peeled potato was taken in a conical flask. Some amount of distilled water was also poured in the flask. Peeled potatoes were then boiled till smell of potato comes out. The potato dextrose broth was collected by straining through cheese cloth and then required amount of dextrose was added. Finally, the volume of medium was adjusted to 100ml. Thereafter medium was sterilized at 15 lb p.s.i. for 15 minutes. At that pressure temperature become 121^0 C.

 # POTATO DEXTROSE AGAR (PDA)

2% agar powder was added to the final potato dextrose broth solution to prepare potato dextrose agar. The agar was melted by heating the media before sterilization at 15 lb p.s.i. for 15 minutes at 121°C.

 # NUTRIENT AGAR (NA)

Beef extract	:	3 g
Peptone	:	10 g
Agar	:	15 g
Distilled water	:	1000 ml
Carbohydrate (if desired)	:	10 g

Required amount of beef extract and peptone were dissolved in distilled water. Agar was then added to the solution and dissolved by heating. Carbohydrate

may be added if required before adding agar. Finally the medium was sterilized at 15 lb p.s.i. for 15 minutes at 121°C.

 # NUTRIENT BROTH (NB)

Beef extract	:	3 g
Peptone	:	10 g
Distilled water	:	1000 ml
Carbohydrate (if desired)	:	10 g

Required amount of beef extract and peptone were dissolved in distilled water. Carbohydrate may be added if required before adding agar. Finally the medium was sterilized at 15 lb p.s.i. for 15 minutes at 121°C.

 # YDC MEDIUM

Yeast extract	:	10 g
Dextrose	:	20 g
$CaCO_3$ (light powder)	:	20 g
Agar	:	20 g
Distilled water	:	1000ml

Dextrose was separately autoclaved at 15 lb p.s.i. for 15 minutes at 121°C in half of the distilled water and other ingredients were also autoclaved in the other half of the distilled water at 15 lb p.s.i. for 15 minutes at 121°C. Finally solutions were combined and stirred before pouring plates on that CaCO3 was evenly distributed (Schaad, 1988).

 # CASAMINO ACID-PEPTONE-GLUCOSE (CPG) MEDIUM

Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glucose	5 g
Agar	17 g

All the ingredients were mixed at proper ratio and pH was adjust at 6.5-7.0 then autoclaved at 15 lb p.s.i. for 20 minutes at 121°C. After autoclave the media was cooled at 50 to 55°C and then poured into petri plates (Denny and Hayward, 2001).

 # TRIPHENY TETRAZOLIUM CHLORIDE (TTC OR TZC) MEDIUM

Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glucose	5 g
Agar	17 g

All the ingredients were mixed at proper ratio and pH was adjust at 6.5-7.0 then autoclaved at 15 lb p.s.i. for 20 minutes at 121°C. After autoclave the medium was allowed to cool at 50 to 55°C and then 5 ml 2, 3, 5-tripheny tetrazolium chloride solution (1% stock solution) was added. Finally the mixed medium was poured into petri plates as suggested by Denny and Hayward, 2001. The stock was also autoclaved for 5 minutes at 121˚C, and stored at 4˚C or frozen.

 # 1% stock solution of 2, 3, 5-tripheny tetrazolium chloride

2,3,5-Triphenyl tetrazolium chloride 0.10 gm and distilled water 10.00 ml (10 ml per vial) was mixed properly and filtered with whatman No. 4 filter paper and finally autoclaved for 5 minutes at 121˚C, and stored at 4˚C or frozen (http://plantpath.ifas.ufl.edu/rsol/Culturemedia.html).

 # PSEUDOMONAS SOLANACEARUM AGAR (PSA) MEDIUM

Peptic digest of animal tissue	10 g
Casein enzymic hydrolysate	1 g
Glucose	5 g
Agar	17 g

All the ingredients were mixed at proper ratio and pH was adjust at 6.5-7.0 then autoclaved at 15 lb p.s.i. for 15 minutes at 121°C. After autoclave the media was cooled at 50 to 55°C and then poured into Petriplates.

7. D. Appendix-IV

Sequences submitted in the GenBank

1. KC237236 Pseudomonas sp. RSG01 16S rRNA

AGGAATCTGCCTGGTAGTGGGGGATAACGTTTAGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGA
AAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATG
GCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGT
GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCT
GTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATCCAAAACTGTCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTG
TAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACA
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA
CTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGG
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGA
ACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGA
GCGCAACCCTTGTCCTTAGTTACCAGCACGTCTTGG

2. KM434237 Ralstonia solanacearum strain RSG02 16S rRNA

TCCACGCGTTAATCGGAAATACAGGCCGTAAAGCGTGCCCAGGCGGTTGAGCAAGACCGATGTGAATTCC
CCCGGCTTAACCTGGGAATTGCATTGGTGACTGCACGGCTAGAGGGTGTCAGAGGGAGGTAGGATTCCTC
GTATCGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGGGAAGGCAGCCTCCTGGGATAACACTG
TCGCTCATGCACGAAACCGTGGAAAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGT
ACACTAGGTGAAGGGGATCCATTTCCTTAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTAC
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ATGCAACCGGAAAAACCTTACCTACCCTTGACATGGGACTAACGAAGCAGAGATGCATTAGGTGGTCGAA
AGAGAAAGTGGACTCACGTGCTGCTTGGCTGTCGTCAGCTCGTGTCGTGTGATGTTGGGTTAAGTCCCCC
AACGAGAGCAAGGCTTGTCTCTAGTTGCTACGAAAGGCGACTCTAGAGAGACTGCCGGTGACAAACCGGA
GGAAGGAGGGGATGACGTCAAGTGGTGATGGCCCTTATGGGTAGGGCTTCCCACGTCATACAATGGTGCA
TACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAATGCATAGTAGTCCGGATCGTCGTCTG
CAACTCGACTACATCAAGCTCCAATCGCTAGT

3. KM434238 Ralstonia solanacearum strain 16S rRNA

TCCACGCGTTAATCGGAATTACAGGGCGTAAAGCGTGCGCAGGCGGTTGAGCAAGACCGATGTGAAATCC
CCGGGCTTAACCTGGGAATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGAGGTAGAATTCCTC
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ACACTAGGTGAAGGGGATCCATTTCCTTAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTAC
GGTCGCAAGATTAAAACTAAAAGGAATTGATGGGGACCCGCAGAAGCGCTGGATGATGTGGATTAATTCG
ATGCAACCGGAAAAACCTTACCTACCCTTGACATGGGACTAACGAAGCAGAGATGCATTAGGTGGTCGAA

AGAGAAAGTGGACTCACGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGTGATGTTGGGTTAAGTCCCGC
AACGAGAGCAAGGCTTGTCTCTAGTTGCTACGAAAGGCGACTCTAGAGAGACTGCCGGTGACAAACCGGA
GGAAGGAGGGGATGACGTCAAGTGGTGATGGCCCTTATGGGTAGGGCTTCCCACGTCATACAATGGTGCA
TACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAATGCATAGTAGTCCGGATCGTCGTCTG
CAACTCGACTACATCAAGCTCCAATCGCTAGT

4. Ralstonia solanacearum strain RSG01 fliC

CTGCAAACGGCCGACTCGTACCTGGGCCAAGTTGAAAACAACCTGCAACGTATGCGCCAGCTGGCTGTGG
AATCCAACAACGGCGGTCTGTCGGCAGCTGACCAGACCAACCTGGACAAGGAATACCAACAGTTGGCAAC
GGCCAACAAGAACATCGAAACCAACGCCAACTACAACGGCAACAAGCTGTTCGACGGCTCGGTGGCTTCG
ACGACCTTCCAATATGGCCAGAACGCAGCCACGGACGTGACCACGGTCACCAACGTCGACATGTCGGCCT
ACGGCACGCTGGCCGGTACGAGCGTGACCAGCGCTGCCAACGCGACCGCAGCCCAGGCCGCGATCGACAC
C

5. Ralstonia solanacearum strain RSG02 fliC

CTGCAAACGGCCGACTCGTACCTGGGCCAGGTTGAAAACAACCTGCAACGTATGCGCCAACTGGCTGTGG
AATCCAACAACGGCGGTCTGTCGGCGGCTGACCAGACCAACCTGGACAAGGAATACCAACAGCTGGCAAC
GGCCAACAAGAACATCGAAACCAACGCCAACTACAACGGCAACAAGCTGTTCGACGGCTCGGTGGCTTCG
ACGACCTTCCAGTATGGCCAGAACGCAGCCACGGACGTGACCACGGTCACCAACGTCAACATGTCGACCT
TCGGCACGCTGACCGGTACGAGCGTGACCAGCGCTGCCAACGCGACCGCAGCCCAGGCCGCGATCGACAC
C

6. Ralstonia solanacearum strain RSG03 fliC

CTGCAAACGGCCGACTCGTACCTGGGCCAAGTTGAAAACAACCTGCAACGTATGCGCCAGCTGGCTGTGG
AATCCAACAACGGCGGTCTGTCGGCAGCTGACCAGACCAACCTGGACAAGGAATACCAACAGTTGGCAAC
GGCCAACAAGAACATCGAAACCAACGCCAACTACAACGGCAACAAGCTGTTCGACGGCTCGGTGGCTTCG
ACGACCTTCCAGTACGGCCAGAACGCAGCCACGGACGCGACCACGGTCACCAACGTCGACATGTCGGCCT
TCGGCACGCTGGCCGGCACGAGCGTGACCAGCGCTGCCAACGCGACCGCAGCCCAGGCCGCGATCGACAC

7. KC959841 Bacillus cereus strain HS01 16S rRNA

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTA
AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG
ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGC
TTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTAC
CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGTGGCAAGCGTTATCCGGA
ATTATTGGCGTAAAGCGCGCGCAGTGGTTTCTTAGTCTGATGTGCTGCCCACGGCTCAACCGTGGAGGGT
CATTGGAAACTGGGAGACTTGATTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
AGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTG
GGGAGCAAACAGGATTAGA

7. E. Appendix-V

List of publications

1. Mandal H., Chakraborty P., Das S., Saha A., **Sarkar T.,** Saha D. and Saha A. (2017) Biocontrol of virulent *Ralstonia solanacearum* isolated by an indigenous *Bacillus cereus. Journal of Agricultural Technology* **13:** 19-30.

2. Saha A., **Mandal H.** and Saha D. (2013).Isolation and identification of a virulent Ralstonia solanacearum by fliC gene amplification and induction of chitinase by 2-amino butyric acid for control of bacterial wilt in tomato plants. NBU Journal of Plant Science **7:** 95-100.

7. F. Appendix-VI:

Reprints

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Biocontrol of Virulent *Ralstonia solanacearum* isolates by an Indigenous *Bacillus cereus*

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Mandal H., Chakraborty, P. S., Saha, D. A., Sarkar, T., Saha, D. and Saha, A. (2017). Biocontrol of virulent *Ralstonia solanacearum* isolates by an indigenous *Bacillus cereus.* International Journal of Agricultural Technology 13(1):19-30.

Abstract In Sub-Himalayan West Bengal tomato is grown round the year. Substantial loss of the crop has been experienced by the farmers' of the area due to wilt disease caused by *Ralstonia solanacearum*. *R. solanacearum* is a devastating, soil borne bacterial pathogen of tomato. The pathogen is non-motile in plant but highly motile in culture. In the present study sixteen strains of *R. solanacearum* were isolated, purified and identified on the basis of physiological and biochemical characteristics. Pathogenicity of all the sixteen isolates was tested and all the isolates were found to be pathogenic.Three of them were virulent and coded as RSG01, RSG02 and RSG03. Identification of most virulent bacterium (RSG01) was also done by 16S rRNA studies. Finally the most virulent pathogen was controlled by an indigenous antagonistic soil bacteria *Bacillus cereus.*

Keywords: *Ralstonia solanacearum*, Wilt, Tomato, *Bacillus cereus*, Antagonism

Introduction

Tomato (*Lycopersicon esculentum* Mill., family Solanaceae) is one of the commercially important vegetable crops. It is rich in minerals, vitamins, organic acids, essential amino acids, dietary fibers, lycopene, beta-carotene etc. and therefore known as protective food. Cultivation of tomato has been increased over the years due to its popularity and economic importance (Elphinstone *et al.*, 1996). During 2012-13, the production of tomato in India was 18,226.6 thousand metric tonnes with a production area of 879.6 thousand hectors. (Anonymous, 2013). The disease problem of tomato especially with *Ralstonia solanacearum* has great importance due to its substantial economic loss making capacity. *R. solanacearum* is a soil born bacterium originated from the tropics, subtropics and warm temperate regions (Hayward, 1991). *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* have been reported to be a wilt disease causing pathogen of solanaceous plants in India (Tans-Kersten *et al.*, 2001). Bacterial wilt is a disease where loss of turgor in plant or plant parts occurs (Windham and Windham, 2004).

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In sub-Himalayan West Bengal (the present study area) tomato is grown round the year and substantial loss of crops has been experienced by the farmers' mainly due to *R. solanacearum*. Considerable efforts have been made to manage the disease in various crops and in different places with the use of host resistance, changed cultural practices, bio agents and chemicals (Dubey *et al.*, 1996; Kisore *et al.*, 1996; Ciampi *et al.*, 1997; Adhikari and Basnyat, 1998; Momol *et al.*, 2000). Chemical and cultural control of this disease in infested soils is a hard task (Grimault *et al.*, 1993). Biocontrol has been proposed to prevent *Ralstonia solanacearum* in many cases (Thongwai and Kunopakarn, 2007).

Hence, in the present study, it was thought to isolate virulent *R. solanacearum* from different wilted tomato plants cultivated in different parts of sub-Himalayan west Bengal, adjoining parts of Assam and Bihar. Isolations were also done from the rhizosphere of tomato plants. In addition it was also thought to find out some indigenous antagonistic organism, if any, to control *R. solanacearum*.

Materials and methods

***Isolation of Ralstonia solanacearum*:** On the basis of bacterial wilt symptoms (observed in the field condition) diseasedplants were selected from the different tomato growing fields of sub-Himalayan West Bengal. Diseased plant samples along with rhizospheric soil were collected for isolation of bacterial pathogens.Bacterial ooze characteristic of bacterial wilt was collected aseptically in sterile distilled water (Fig-1). Ooze solution (1 ml) was mixedwith20 ml of nutrient broth and finally poured into a Petri plate of 90mm in diameter. Bacteria were also isolated from rhizospheric soil by using soil dilution techniqueand agar plate method as suggested by Dhingra and Sinclair (1995). *Pseudomonas solanacearum* specific agar medium was routinely used for maintaining the isolates.

***Pathogenicity test*:** Pathogenicity test of the bacterial pathogens were performed following the method of Hoque and Mansfield, 2005. Healthy (10 cm long) tomato plants [Var. PKM1] weretransplanted into plastic pots (size: 15 cm diameter × 15 cm in length) containing sterilized garden soil. All the bacterial strains to be tested were freshly cultured in nutrient broth medium. Twenty milliliter of half diluted culture was mixed with the soil surrounding the test plant. In case of control 20 ml of sterilized water was added instead of half diluted culture. Percent disease symptom of wilt was recorded after 3rd day ofinoculation till 12thday.

Figure 1. Bacterial Ooze coming out in clear water from cut end of a wilt infected tomato stem.

Screening and isolation of antagonistic microorganism: Screening and isolation of antagonistic microorganisms were done following the method of Lwin and Ranamukhaarachchi (2006). Rhizospheric soil sample (10g) was mixed with 100ml of sterilized water. A serial dilution was made upto 10^4 dilution factor.Sterilized nutrient agar platesware inoculated by the 0.1 ml soil solution of 10^{-4}dilutions. After, 72 hours of incubation at 30ºC, a large bacterial colony intermingled with several other colonies was found to inhibit the growth of the other bacterial colonies. On the basis of preliminary antagonism shown, the bacteria was isolated carefully and used for tests against the pathogenic bacteria. Theantagonistic bacterium was sub-cultured in nutrient agar medium. All such bacteria isolated were observed under light microscope to confirm the shape of each bacterium.

Biochemical characterization: The classical approach to bacterial identification involves preliminarily microscopic examination following Gram reaction which divides bacteriainto two broad groups (Gram + ve and Gram – ve). In addition several biochemical tests were performed to identify the bacteria up to the genus level as suggested by Trigiano *et al.* (2004). The

major biochemical experiments performed were Gram staining, suitable growth condition (aerobic/anaerobic) on common laboratory media (Nutrient agar & nutrient broth), Yellow pigmentation on YDC medium, Growth on D1M medium, Growth above 40ºC, Growth bellow 4ºC, Oxidase test and finally different carbohydrate utilization test with KB009 HiCarbohydrate™ Kit.

Scanning Electron Microscopy: One sterile cover glass was placed in a sterile Petriplate and a bacterial smear was prepared on that coverglass. The cover glass was subjected to series of treatment prior to observation under scanning electron microscope (SEM). The process of Samaranayake *et al.* (2005) was followed for treating the bacterial smear on cover glass. The bacteria were fixed with 2.5% glutaraldehyde solution for one hour. Glutaraldehyde was removed by decanting. Then dehydration of the material was done by an ascending series of ethanol. After dehydration the samples were coated with gold (IB2-ion coater, Japan) and observed under scanning electron microscope [Model: Hitachi S-530(Japan) 1986].

DNA extraction and PCR amplification: The method of Ausubel *et al.* (1992) was followed for the isolation of genomic DNA. Polymerase chain reaction (PCR) was performed using three sets of 16S rDNA primers (Table-1) as suggested by Boudazin *et al.* (1999) for identification of pathogenic *Pseudomonas* and antagonistic *Bacillus* sp. Amplicons were analyzed by electrophoresis in 0.8% agarose gel. Finally the expected amplicons were sent for sequencing to Xcelris Genomics Ltd., annotation and BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) analysis and submitted to GenBank.

Table 1. Primers used in the study

Primer 1	Forward	5′-GAGTTTGATCATCATCGCTCAG-3′
	Reverse	5′-GGCGGGACTTAACCAACAT-3′
Primer 2	Forward	5′-GTCCGGAAAGAAATCGCTTC-3′
	Reverse	5′-CCAGTCATGAACCCTACGTG-3′
Primer 3	Forward	5′-AGAGTTTGATCCTGGCTCAG-3′
	Reverse	5′-TACGGTTACCTTGTTACGACTT-3′

Results

Sixteen different bacteria (*Ralstonia solanacearum*) were isolated from either wilted tomato plants or from rhizospheric soil of the infected tomato plants of sub-Himalayan West Bengal. All the sixteen isolates were subjected to identification by morphological and biochemical characterization along with ooze test (Windham and Windham, 2004). On the basis of biochemical characterization it was found that all the sixteen bacteria were Gram negative, aerobic and oxidase positive. But they were negative in all other biochemical tests performed (anaerobic growth, pigmentation on YDC medium, growth bellow 4°C and above 40°C and

growth in D1M medium). Biochemical test results of three selected bacteria have been shown in Table-2. All the sixteen bacteria were found to be pathogenic but the degree of pathogenicity varied greatly. Three most virulent bacteria were selected and then subjected to pathogenicity test again. Wilting index was calculated on the basis of five point scale [- = no disease, + = drooping of the leaves up to 20%, ++ = Drooping of plants between 21 - 40%, +++ = Drooping of plants between 41 - 60%, ++++ = 61 - 80%. +++++ = 100% drooping and wilting of plants].From the results of pathogenicity test it was evident that 'RSG01' was most virulent (Table-2).From the results it was also evident that *R. solanacearum* (RSG01) was most virulent and within 12 days the plants were almost devastated by wilting (Wilting index = +++++). The other two pathogens (RSG02 and RSG03) were moderately pathogenic and the plants inoculated by those pathogens showed wilting index of '+++' indicating 41- 60% wilting (Table-3).

Table 2. Biochemical characterization of the three pathogenic *Ralstonia solanacearum* isolates.

Experiments	**RSG01**	**RSG02**	**RSG03**
Gram staining	(-)ve	(-)ve	(-)ve
Aerobic growth	+	+	+
Anaerobic growth	-	-	-
Yellow pigmentation on YDC medium	-	-	-
Growth above 40°c	-	-	-
Growth bellow4°c	-	-	-
Growth on D1M medium	-	-	-
Oxidase test	+	+	+

'+'= Growth ; '-'= No growth

Table 3. Pathogenicity of the three virulent isolates of *Ralstonia solanacearum.*

***Ralstonia solanacearum* isolates**	**Wilting index* (days after inoculation)**			
	3 d	6 d	9 d	12 d
RSG01	+	++	+++	+++++
RSG02	-	+	++	+++
RSG03	-	+	++	+++
Control (sterile distilled H_2O)	-	-	-	-

*Wilting index was calculated on the basis of a 5 point scale. [- = no disease, + = drooping of the leaves up to 20%, '++'= Drooping of plants between 21- 40%, '+++'= Drooping of plants between 41-60%, '++++'= 61-80%. '+++++'= 100% drooping and wilting of plants.]

All the bacteria were observed in light microscope. Shape of all the bacteria was found to be rod. The most virulent *R. solanacearum* (RSG01),was also observed under scanning electron microscope to understand the surface topography of the bacterium (Fig-1).Tomato plants [Var. PKM1] were artificially infected by *R. solanacearum* (RSG01) and after six days ooze test were performed. From the ooze test it was evident that substantial amount of ooze was coming out from the cut end of the stem (Fig-2). This indicated that *R. solanacearum* (RSG01) was a virulent pathogen.

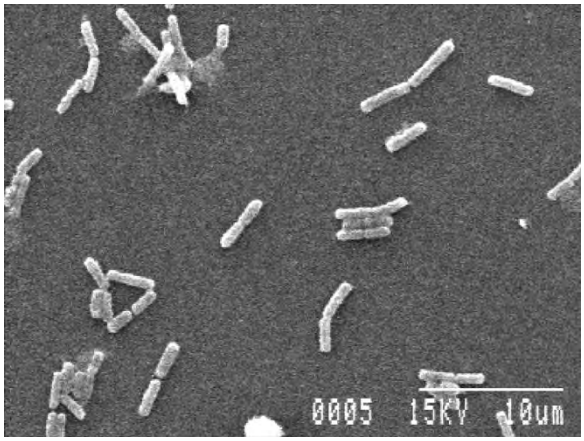

Figure 2. Scanning Electron Microscopic image of RSG01.

R. solanacearum (RSG01) and the other two virulent bacteria (RSG02 and RSG03) were selected and were subjected to carbohydrate utilization tests. Altogether, 33 carbohydrates and their derivatives were used for the test. All the three virulent isolates could use only thirteen carbohydrates (Ramnose, Cellobiose, Xylitol, ONPG, D-Arabinose, Citrate, Malonate, Xylose, Fructose, Dextrose, Galactose, Malibiose and L-Arabinose) among the tested carbohydrates. Other twenty carbohydrates (Melizitose, Methyl-D-Manoside, Esculin, Solbitol, Lactose, Raffinose, Maltose, Trehalose, Sucrose, Inuline, Sodium glucanate, Glycerol, Salicin, Dulcitol, Inocitol, Sorbitol, Mannitol, Adonitol, Arbitol and Erythritol) tested could not be used by any of the three bacteria. Identification of the bacteria was performed by comparing with the characters of the present study with that of stated in the Bergey's manual of systematic bacteriology, 9^{th}edition.

***Host range test*:** In order to check infectivity of the three bacteria, potato, another solanaceous plant, was taken into consideration. Results of the infectivity of the bacterium in potato in comparison to tomato were observed. The results indicated that all the three isolates (showed virulence to tomato) were capable to infect potato but they were less virulent in potato than in tomato as evidenced by the results presented in the form of wilting

index (Table-4).The RSG01 strain was found to be more virulent than the other two strains both in potato and tomato plants.

Table 4. Host infectivity of the bacteria on two solanaceous plants.

Host	RSG01	RSG02	RSG03
Tomato plant	+++	++	++
Potato plant	++	+	+

*Wilting index was calculated on the basis of a 5 point scale. ['+'= drooping of the leaves up to 30%, '++'= Drooping of plants between 31- 60%, '+++'= Drooping of plants between 61-90%.]

***Evaluation of antagonism*:** One bacterial isolate (HS01) was found from the rhizosphere soil of wilted tomato field. The bacterium showed antagonism in mixed soil bacterial culture in Petriplate. The bacterium was isolated in pure form and was subjected to cross culture following the method of Dhingra and Sinclair (1995) in nutrient agar medium. Result showed that the most virulent isolate could not grow at the cross point but the antagonist could grow at that point (Fig-3). From the result it was evident that the bacterium (HS01) was antagonistic against virulent *Ralstonia solanacearum* (RSG01).

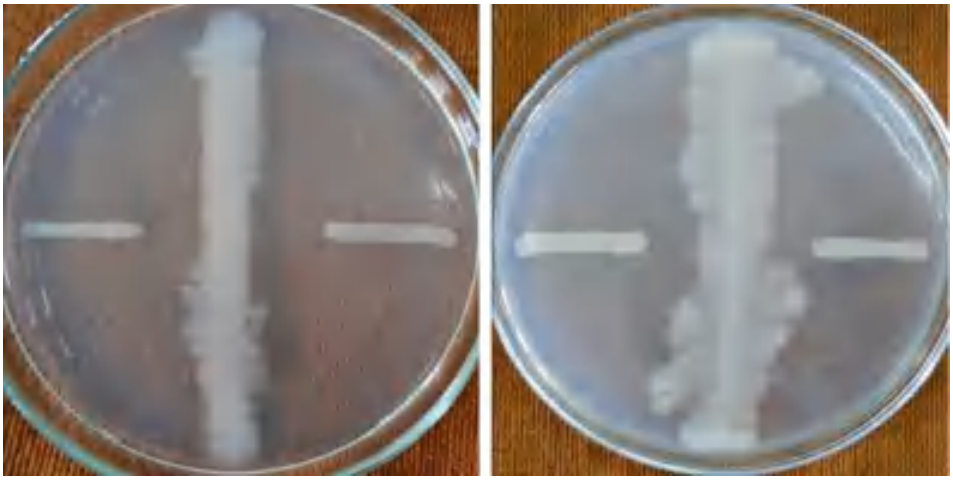

Fig-3: a) Dual culture of *Bacillus cereus* and *Ralstonia solanacearum* isolate RSG01 and b) Dual culture of *Bacillus cereus* and *Ralstonia solanacearum* isolate RSG02 .

***16SrRNA studies for identification of bacteria*:** One virulent and one antagonistic bacterium (RSG01 and HS01) were subjected to molecular identification following 16S rRNA studies. Initially, expected amplicons raised through PCR using suitable primer pairs were subjected to agarose gel electrophoresis (Fig-4). The size of the amplicons was 1016 bp long for *R. solanacearum* (virulent isolate RSG01) and 789 bp long for *Bacillus cereus* (antagonistic isolate HS01) (Fig-5). The 16S rRNA sequences of

both the bacteria were submitted in the GenBank for accession. The sequences received from the GenBank were analyzed by BLAST. The virulent bacterium was identified as *Ralstonia solanacearum* while the antagonist was identified as *Bacillus cereus*. The GenBank accession numbers of the two bacteria are KC237236 and KC959841 respectively.

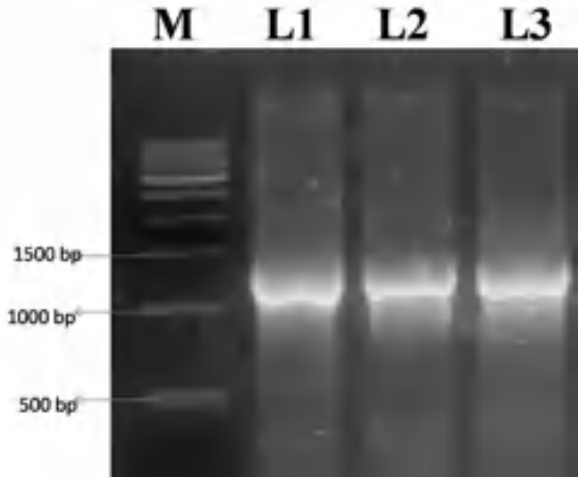

Figure 4. Expected amplicons (raised through PCR using suitable 16s rRNA primer pairs) of *R. solanacearum* isolates on agarose gel. M= 500bp ladder; L1, L2, L3= RSG01, RSG02, RSG03 isolates respectively.

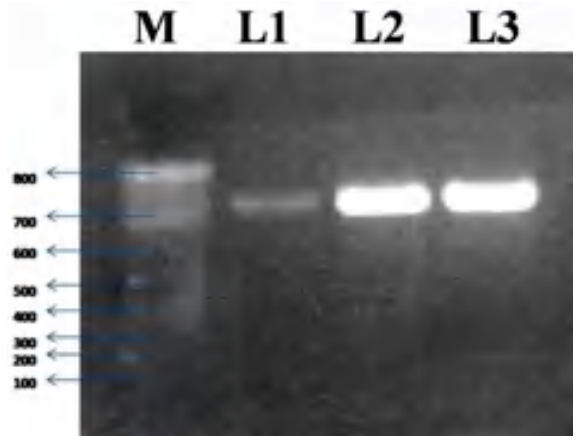

Figure 5. Expected amplicons (raised through PCR using suitable 16s rRNA primer pairs) of *Bacillus cereus* isolate on agarose gel. M= 100bp DNA ladder; L1, L2 and L3= HS01 isolate

Discussion

Bacterial wilt by *Ralstonia solanacearum* is one of the devastating diseases of tomato in the world (Chandrashekara *et al.*, 2012). Sixteen different bacteria causing wilt disease in tomato in sub-Himalayan West

Bengal were isolated. All such bacteria were found to produce bacterial ooze. A cross section of the stem of a plant showing bacterial wilt may produce white, milky strands of bacterial cells in clear water. This ooze distinguishes the wilt caused by bacterium from that caused by fungal pathogens (Leppla *et al.*, 2004; Hernandez-Romano *et al.*, 2012).Tomato is a major horticultural crop of sub-Himalayan West Bengal. The area is popularly known as North Bengal. It has been reported that the incidence of bacterial wilt in tomato crops in India ranges from 15 to 55% and the disease causes 25 to 75% yield loss of solanaceous vegetables (Rao and Sohi 1977). In our study, also it has been experienced that about 20% of the plants in the fields, where proper control measures were not taken, were attacked by pathogenic bacteria *Ralstonia solanacearum*. According to Chandrashekara *et al.* (2012) the virulence of *R. solanacearum* may be of great use for managing the pathogen.

Bacterial wilt pathogens of the infected samples were isolated and purified. Some other *Ralstonia solanacearum* were also isolated from the rhizospheres of the tomato plants which were severely affected by bacterial wilt. Sixteen different *Ralstonia solanacearum* were isolated, purified and identified on the basis of physiological and biochemical characteristics. Identification of the bacteria on the basis of physiological and biochemical characteristics were performed following the flowchart identification scheme of Trigiano *et al.* (2004). Study of the bacteriological properties of the isolates confirmed that the isolates were aerobic and gram-negative. The notions of our study of bacterial properties were similar with that of Ozaki and Watabe (2009).

All the sixteen isolates from infected tomato plants or tomato rhizosphere were subjected to pathogenicity test, which showed that all the sixteen isolates were differentially pathogenic to tomato plants but three of them were virulent and highly pathogenic. Those three isolates were coded as RSG01, RSG02 and RSG03. Pathogenicity of the three isolates was also studied in potato plants to know their capability of infection in a related crop of the same family solanaceae. All the three isolates could infect both potato and tomato. Pathogenicity test of *R. solanacearum* isolates of geranium and portulaca were also studied by Ozaki and Watabe (2009) in different hosts to know their infectious capacity.

Identification of most virulent bacterium (RSG01), of the present study, was also done by 16S rRNA studies following BLAST analysis. A number of studies have supported the molecular identification of *Ralstonia solanacearum* following 16S rRNA studies (Fouche-Weich *et al.*, 2006).The pathogen (RSG01) was also controlled by an indigenous antagonistic bacterium isolated from the soil and identified as *Bacillus cereus* HS01 by 16S rRNA study and BLAST analysis. The antagonism of the bacterium was studied following dual culture technique as suggested by several workers (Yoshida *et al.,* 2001; Romero *et al.*, 2004; Thongwai and

Kunopakarn, 2007). Antagonism of other *Bacillus* sp. and several other bacterial antagonists towards phytopathogens has been demonstrated by many scientists. (Manjula and Podile, 2005; Seleim *et al.*, 2011; Singh *et al.*, 2012; Chen *et al.*, 2013; Maji and Chakrabartty, 2014; Singh and Siddiqui, 2015).

Thus, from the present study it may be concluded that the virulent *R. solanacearum* isolates of the present study area are pathogenic to tomato and potato. One antagonistic bacterium against virulent pathogenic *R. solanacearum* isolates are available in the fields of the present study area and may be exploited for development of suitable formulations for use in the fields to control *R. solanacearum* to get rid of bacterial wilt of tomato.

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References

Adhikari, T. B. and Basnyat, R. C. (1998). Effect of crop rotation and cultivar resistance on bacterial wilt of tomato in Nepal. Canadian Journal of Plant Pathology 20: 283-287.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J, A. and Struhl, K. (1992). Current protocols in molecular biology, v.1. Greene Publishing Association, New York.

Boudazin, G., Leroux, A. C., Josi, K., Labarre, P. and Jouan, B. (1999). Design of divisions specific primars of *Ralstonia solanacearum* and application to the identification of European isolate. European Journal of Plant Pathology 105:373-380.

Chandrashekara, K. N., Prasanna Kumar, M. K. and Saroja, S. (2012). Aggressiveness of *Ralstonia solanacearum* isolates on tomato. Journal of Experimental Sciences 3: 5-9.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R. and Guo, J. H. (2013). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environmental Microbiology 15:848-864.

Ciampi, L., Burzio, L. O. and Burzio, L. A. (1997). Carriers for *Pseudomonas* (*Ralstonia*) *solanacearum*, causal agent of bacterial wilt. Fitopathologia 32:64-70.

Dhingra, O. D. and Sinclair, J. B. (1995). Basic Plant Pathology Methods, 2nd ed. CRC Press, London.

Dubey, L. N., Das, K. K. and Hazarika, D. K. (1996). Evaluation of some chemicals against bacterial wilt of *Sesamum*. Journal of Mycological Plant Pathology. 26:94-95.

Elphinstone, J., Hennessy, J., Wilson, J. and Stead, D. E. (1996). Sensitivity of different methods for the detection of *Pseudomonus solanacearum* (Smith) Smith in potato tuber extracts. EPPO Bulletin 26:663-678.

Fouche-Weich, J., Poussier, S., Trigalet-Demery, D., Berger, D. and Coutinho, T. (2006). Molecular identification of some African strains of *Ralstonia solanacearum* from eucalypt and potato. Journal of General Plant Pathology 72:369-373.

Grimault, V., Schmit, J. and Prior, P. (1993). Some characteristics involved in bacterial wilt (*Pseudomonas solanacearum*) resistance in tomato. *In*: Hartman GL, Hayward (eds). Bacterial wilt. ACIAR Proceedings 112-119.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt, caused by *Ralstonia solanacearum*. Annu. Rev. Phytopathol 29: 65-87.

Hernandez-Romano, J., Ramirez-Rojas, S. and Ydrac-Morales, C, J. (2012). First report of *Ralstonia solanacearum* causing tomato bacterial wilt in Mexico. New Disease Reports 26:22.

Hoque, M. E. and Mansfield, J. W. (2005). A sample and reliable method for pathogenicity tests of bacterial blight disease of rice. Bangladesh Journal of Botany 34:11-16.

Kisore, V., Shekhawat, G. S. and Sunaina, V. (1996). Cultural practices to reduce *Pseudomonas solanacearum* in the infested soil. Journal of Indian Potato. 23:130-133.

Leppla, N., Momol, T., Nesheim, N. and Dusky, J. (2004). Plant, animal and human protection, FAS 2 Focus Area. University of Florida / IFAS. Available at: http://edis.ifas.ufl.edu/.

Lwin, M. and Ranamukhaarachchi, S. L. (2006). Development of biological control of *Ralstonia solanacearum* through antagonistic microbial populations. International Journal of Agriculture Biology 5:657-660.

Maji, S. and Chakrabartty, P. K. (2014). Biocontrol of bacterial wilt of tomato caused by *Ralstonia solanacearum* by isolates of plant growth promoting rhizobacteria. Australian Journal of Crop Science 8:208-214.

Manjula, K. and Podile, A. R. (2005). Increase in seedling emergence and dry weight of pigeon pea in the field with chitin-supplemented formulations of *Bacillus subtilis* AF1. World Journal Microbiological Biotechnology 21:1057-1062.

Momol, M. T., Mitchell, D. J., Rayside, P. A., Olson, S, M. and Momol, E. A. (2000). Plant essential oils as potential biofumigants for the management of soilborne pathogens of tomato. Phytopathology 90:S127.

Anonymous (2013). Indian Horticulture Database 2013. National Horticulture Board, New Delhi, India.

Ozaki, K. and Watabe, H. (2009). Bacterial wilt of geranium and portulaca caused by *Ralstonia solanacearum* in Japan. Bull. Minamikyushu Univ. 39:67-71.

Rao, M. V. B. and Sohi, H. S. (1977). Control of bacterial wilt of brinjal. Indian Horticulture 22:11-13.

Romero, D., Peerez-Garcia, A., Rivera, M, E., Cazorla, F. M. and de Vicente, A. (2004). Isolation and evaluation of antagonistic bacteria towards the curcurbit powdery mildew fungus *Podosphaera fusca*. Applied Microbiological Biotechnology 64:263-269.

Samaranayake, Y, H., Ye, J., Yau, J. Y., Cheung, B. P. and Samaranayake, L. P. (2005). *In vitro* method to study antifungal perfusion in *Candida* biofilms. Journal Clinical Microbiology 43:818-825.

Seleim, M. A. A., Saead, F. A., Abd-El-Moneem and Abo-Elyousr, K. A. M. (2011). Biological control of bacterial wilt of tomato by plant growth promoting rhizobacteria. Plant Pathology Journal 10:146-153.

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

Singh, N. and Siddiqui, Z. A. (2015). Effects of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Aspergillus awamori* on the wilt-leaf spot disease complex of tomato. Phytoparasitica 43:61-75.

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

Thongwai, N. and Kunopakarn, J. (2007). Growth Inhibition of *Ralstonia solanacearum* PT1J by antagonistic bacteria isolated from soils in the northern part of Thailand. Chiang Mai Journal of Science 34:345-354.

Trigiano, R. N., Windham, M. T. and Windham, A. S. (2004). Plant Pathology: Concepts and Laboratory Exercises. 5th ed. CRC Press, Boca Raton, FL, USA.

Windham, M. T. and Windham, A. S. (2004). Plant Pathology and Historical Perspectives. *In*: Trigiano RN, Windham MT, Windham A. S. (eds.) in Plant Pathology: Concepts and Laboratory Exercises. 5th ed. CRC Press, Boca Raton, FL, USA, pp. 14-15.

Yoshida, S., Hiradate, S., Tsukamato, T., Hatakeda, K. and Shirata, A. (2001). Antimicrobial activity of culture filtrate *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 91:181-187.

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Isolation and identification of a virulent *Ralstonia solanacearum* by *fliC* gene amplification and induction of chitinase by 2-amino butyric acid for control of bacterial wilt in tomato plants

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Abstract

Ralstonia solanacearum is a devastating, soil borne bacterial pathogen of tomato. The pathogen is nonmotile in planta but highly motile in culture. On the basis of physiological and biochemical characteristics 26 isolates have been purified and identified as *Ralstonia solanacearum*. The *fliC* gene is responsible for the movement of bacteria. *Ralstonia* specific *fliC* gene amplification is the indication of virulence of the pathogen. In the present study one *R. solanacearum* isolate has been identified by PCR amplification of the *fliC* gene using *fliC* gene specific primer. Following isolation and identification of the virulent isolate, fresh tomato plants were induced by application of 2-amino butyric acid (ABA). The defense enzyme, chitinase was estimated in treated plants. Treated inoculated plants did not show any visible symptoms of wilt even after 14 days of inoculation. Significantly it was observed that chitinase was increased in the 2-ABA-treated plants and also in the treated-inoculated plants. The increased chitinase activity in the treated plants showed that 2-ABA has the resistance inducing capacity in tomato plants against *Ralstonia solanacearum*.

Keywords: isolation, *Ralstonia solanacearum*, *fliC* gene, chitinase, 2-ABA.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most economically important vegetable crop and it is cultivated worldwide for its freshly fruits and economic importance (Elphinstone *et al*., 1996). The causal organism of bacterial wilt is *Ralstonia solanacearum*. *R. solanacearum* is a soil born bacterium originating from the tropics, subtropics and warm temperate regions (Hayward, 1991). *R. solanacearum* is a serious pathogen causing bacterial wilt in solanaceous vegetables in India, such as tomato, potato, banana, eggplants and some ornamental plants (Tans-Kersten *et al*., 2001).

Control of bacterial wilt is difficult and it may be done by using resistant cultivars. Chemical control is not suitable and use of fumigants is of limited use. Biological control has also been suggested by some authors (Goolmar and Conrath, 2008).

In the genomic DNA of the bacterium the *fliC* gene is found. *fliC* gene is responsible for the production of flagellin protein, which has a major role in the movement of bacteria. Presence of *fliC* gene also indicates the virulent nature of the bacteria (Pfund *et al*., 2004). Hence, if amplification of the *fliC* gene could be done by using *fliC* gene primers, the virulent nature of the isolate may be ascertained. The present work has been undertaken to isolate virulent *Ralstonia solanacearum* isolates from infected plants of north Bengal by PCR amplification of *fliC* gene visible on the agarose gel. After identification of a virulent bacterium, its management becomes necessary. The most virulent isolate of the present study, therefore, controlled by inducing defense enzyme, chitinase, in susceptible tomato plants by 2-ABA.

MATERIALS AND METHODS

Survey of different diseased tomato fields of North Bengal

The infected plant samples were collected from different tomato growing fields of North Bengal. The three districts (Uttar Dinajpur, Dakshin Dinajpur and Cooch Behar) were chosen for the present study.

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Isolation of bacterial isolates from diseased plants

The infected plant samples were collected and brought to the laboratory for the isolation of the bacteria. Then all plant samples were washed with distilled water and a cross section of each diseased plant stem was made, which produced white, milky layer of bacterial cells (bacterial ooze) in clear sterile distilled water (Fig. 1). Then the water was used as sample for isolation of bacteria (Leppla *et al.*, 2004) 0.1 ml of bacterial suspension was spread

Figure 1. Bacterial ooze coming out from the cut end of stem of infected tomato plants.

on *Pseudomonas solanacearum* agar medium and incubated at 32° C for 24 hours. After the incubation the bacterial isolates were subcultured in fresh *Pseudomonas solanacearum* agar medium. All together 26 such samples were found to be pure and kept in the laboratory with proper code for further use in future.

Biochemical characterization

According to the Lacy and Lukezic, (2004) several biochemical tests were performed. The biochemical tests were Gram staining, anaerobic growth, yellow pigmentation on YDC medium, growth above 40°C, growth below 4°C, growth on D1M medium and oxidase test.

DNA extraction and PCR amplification

The method of Ausubel *et al.*, (1992) was followed for the isolation of genomic DNA. Polymerase chain reaction (PCR) was performed using a set of *fliC* gene primer as suggested by Schonfeld *et al.*, (2003).

Primer pair: Rsol_*fliC* forward 5'-GAACGCCAACGGTGCGAACT-3'

and reverse 5'-GGCGGCCTTCAGGGAGGTC-3'.

Assay of chitinase

The colorimetric assay of chitinase was carried out according to the procedure developed by Mahadevan and Sridhar, (1982). Colloidal chitin was prepared as per the method of Berger and Reynolds, (1958). One gram of tomato leaves were extracted in 5.0 ml of 0.1M sodium acetate buffer, pH 5.2 containing 700mg of PVP using mortar and pestle in cold condition. The homogenate was filtered by using four layered muslin cloth, centrifuged at 10000g for 10 minutes and the supernatant was used as crude enzyme source. The assay mixture consisted of 0.5ml crude enzyme, 0.25ml of 0.1M sodium acetate buffer, pH 5.2 and 1ml colloidal chitin (1.8mg/ml) incubated at 37°C for 2h. One ml of reaction mixture was taken and then 1ml of distilled water was added to it. The mixture was boiled for 10 minutes and centrifuged at 5000g for 3 minutes to stop the reaction. One ml of the supernatant was added to 0.1ml of 0.8M potassium tetra borate and boiled exactly for 3 minutes. Then hot mixture was cooled and added to 3ml of para-di-methyl amino benzaldehyde (DMAB) reagent. Samples were incubated again at 37°C for 20 minutes. Immediately after incubation the mixture was cooled and absorbance was recorded within 10 minutes at 585 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis ($\mu mol\ min^{-1}\ g^{-1}$) using N-Acetyl-D Glucosamine as standard.

RESULTS AND DISCUSSION

On the basis of visual observation, 26 bacterial-wilt affected samples were collected from three districts of North Bengal. All the samples were

brought to the laboratory and bacteria were isolated from each sample following the technique as described in materials and methods. All the bacteria were coded. After the isolation of the bacteria, they were allowed to infect tomato plants grown in the experimental plots. Pathogenicity of each bacterium was separately assessed and the wilting index was calculated on the basis of a five point scale. Results of the pathogenicity have been presented in table-1. From the results (Table-1) it was evident that *R. solanacearum* (isolate T_4) of Haldibari of Coochbehar district was most virulent and caused complete wilting (wilting index = +++++) of the test plants. Thirteen isolates showed least wilting index (+). Twelve isolates showed moderate wilting.

Table 1: Isolation of bacteria from infected plants of the places studied

Sr. No.	Place of collection of diseased samples	Isolate code	Pathogenicity status (Wilting index)
1	Haldibari (Cooch Behar)*	T_1	++
2	Haldibari (Cooch Behar)	T_2	++
3	Haldibari (Cooch Behar)	T_3	+
4	Haldibari (Cooch Behar)	T_4	+++++
5	Haldibari (Cooch Behar)	D_1	++
6	Haldibari (Cooch Behar)	D_2	+
7	Haldibari (Cooch Behar)	D_3	++
8	Haldibari (Cooch Behar)	D_4	++
9	Haldibari (Cooch Behar)	D_5	+
10	Haldibari (Cooch Behar)	D_6	+
11	Dumaser (Uttar Dinajpur)	A_1	+
12	Dumaser (Uttar Dinajpur)	A_2	++
13	Dumaser (Uttar Dinajpur)	A_3	+
14	Dumaser (Uttar Dinajpur)	A_4	*
15	Balurghat (Dakshin Dinajpur)	G_1	+
16	Balurghat (Dakshin Dinajpur)	H_1	+
17	Balurghat (Dakshin Dinajpur)	H_4	++
18	Balurghat (Dakshin Dinajpur)	H_5	++
19	Balurghat (Dakshin Dinajpur)	H_6	+
20	Balurghat (Dakshin Dinajpur)	H_7	+
21	Balurghat (Dakshin Dinajpur)	H_8	++
22	Balurghat (Dakshin Dinajpur)	H_9	++
23	Balurghat (Dakshin Dinajpur)	H_{10}	++
24	Gholamdanga (Cooch Behar)	B_1	+
25	Gholamdanga (Cooch Behar)	B_2	+
26	Gholamdanga (Cooch Behar)	B_3	++

*Names given in parenthesis are the name of the districts of the collection spot.

Biochemical characterization

All the isolates were subjected to biochemical characterization for identification. Results of the Gram reaction showed that all the 26 bacteria were Gram negative. From the other results (Table-2) it was also evident that all the bacteria were aerobic. Two bacteria (isolate B_3 and D_4) could produce pigment on YDC medium and the other 24 bacteria were unable to produce pigment. Only two bacteria (isolate D_4 and H_4) could grow at 40° C and above. But, no bacteria could grow at 4°C. One bacterium (isolate D_4) could grow on DIM medium. Out of the bacteria tested three bacterial isolates (isolate H_4, H_5 and A_2) were oxidase negative. The above results were compared with that of given in Bergey's Manual of systematic bacteriology. Vol 1, section 4. Compared results suggested that all the 26 bacteria were *Ralstonia solanacearum*. Our

Table 2: The biochemical characteristics of the isolated bacteria

Isolates in code	Biochemical tests performed						
	Gram staining	Anaerobic growth	Yellow pigmentation on YDC media	Growth above 40°c	Growth below 4°c	Growth on D1M medium	Oxidase test
S_1	-	-	-	-	-	-	+
S_2	-	-	-	-	-	-	+
S_3	-	-	+	-	-	-	+
D_1	-	-	-	-	-	-	+
D_2	-	-	-	-	-	-	+
D_3	-	-	-	-	-	-	+
D_4	-	-	-	+	-	+	+
D_5	-	-	+	-	-	-	+
D_6	-	-	-	-	-	-	+
G_1	-	-	-	-	-	-	+
H_1	-	-	-	-	-	-	+
H_4	-	-	-	-	-	-	-
H_5	-	-	-	+	-	-	-
H_6	-	-	-	-	-	-	+
H_7	-	-	-	-	-	-	+
H_8	-	-	-	-	-	-	+
H_9	-	-	-	-	-	-	+
H_{10}	-	-	-	+	-	-	+
T_1	-	-	-	-	-	-	+
T_4	-	-	-	-	-	-	+
T_5	-	-	-	-	-	-	+
T_6	-	-	-	-	-	-	+
A_1	-	-	-	-	-	-	+
A_2	+	-	+	-	-	-	+
A_3	-	-	+	+	-	-	-
A_4	+	-	+	-	-	-	+

results were also compared with the flow chart identification of of genera of phytopathogenic bacteria as suggested by Lacy and Lukezic,2004

Confirmation of virulence of the bacterial isolate T6 of *R. solanacearum*

Virulance of *R. solanacearum* has been correlated with the presence of *fliC* gene in the bacterium. In the present study the presence of the gene has been confirmed by amplification of the *fliC* gene by PCR and visualization of the amplicon of the expected size (. 550bp) on agarose gel after electrophoresis (Fig. 2). The expected amplicon confirmed the virulence of the bacterium shown during pathogenicity test. The presence of *fliC* gene in the present virulent isolate are in conformity with that of Pfund *et al.*, (2004) who reported that for full virulence flagellin, a product of *fliC* gene is required.

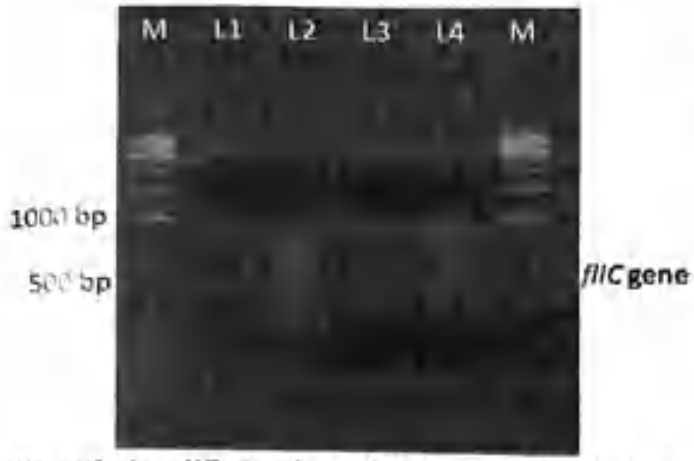

Figure 2. Amplified prduct of *fliC* gene of the virulent isolate T_6 on agarose gel after electrophoresis.

Induction of defense enzyme: chitinase

Many phytopathogenic fungi contain chitin as major structural cell wall component (Wessels and Sietsma, 1981). Chitinases commonly known as plant hydrolases are the key defense enzyme for plant protection against fungal pathogens. Several bacteria were also controlled by induction of chitinase in host plants. Although chitinase degrade chitin present in fungal wall components and several authors have demonstrated the activity of chitinase as growth inhibitor of fungi (Arlorio *et al.*, 1992; Mauch *et al.*, 1988), but the mechanism of controlling bacterial pathogen are yet to be known. In the present study the defense enzyme chitinase have been induced by 2-ABA treatment. From the results presented in Table 3 it was evident that inoculated plants showed maximum chitinase activity (22mg GlcNAc g^{-1} tissue h^{-1}) at 9th day but 2-ABA treated plants showed maximum activity (60mg GlcNAc g^{-1} tissue h^{-1}) after the same period of treatment. When simultaneous treatment and inoculation were done more chitinase activity (65mg GlcNAc g^{-1} tissue h^{-1}) were observed. In distilled water treated plants chitinase activity was recorded as 14mg GlcNAc g^{-1} tissue h^{-1}. From the results it may be concluded that induction of chitinase activity is related to control of the bacterial pathogen in tomato. Chitinase has been reported to control *R. solanacearum* and some other Gram negative bacteria by agglutination (Guan *et al.*, 2008). Thus increased level of chitinase in plants inhibits the entry of the virulent bacterial pathogens in plant cells and thus protects the plants.

Table 3: Chitinase activity in tomato (variety PKM 1) pretreated with 2-ABA followed by challenge inoculation of *Ralstonia solanacearum*

Plant treatment	Chitinase activity (mg GlcNAc g^{-1} tissue h^{-1})			
	Days after treatment and /or inoculation			
	3 Days	6 Days	9 Days	12 Days
Pathogen inoculated	12	15	22	20
Treated with 2-ABA	30	47	60	55
Plants pretreated with 2-ABA and inoculated with *R. solanacearum*	35	55	65	60
Plants sprayed with distilled water (Control)	0.5	0.8	14	10

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References

Arlorio M, Ludwig A, Boller T and Bonfante P. 1992. Inhibition of fungal growth by plant chitinases and β-1,3-glucanases: A morphological study. *Protoplasma* 171:34-43.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K.1992. Current protocols in molecular biology, v.1, Green Publishing Association, New York.

Berger LR and Reynolds DM.1958. The chitinase system of a starin of *Streptomyces griseus*. *Biochem. Biophys. Acta.* 29:522-534.

Elphinstone J, Hennessy J, Wilson J and Stead DE. 1996. Sensitivity of different methods for the detection of *Pseudomonus solanacearum* (Smith) Smith in potato tuber extracts. *EPPO Bulletin.* 26: 663-678.

Goellner K and Conrath U. 2008. Priming: it's all the world to induced disease resistance. *Eur J Plant Pathol* 121: 233-242.

Guan Y, Ramalingam1 S, Nagegowdal D, Taylor PWJ and Chye1 Mee-Len. 2008. Brassica juncea chitinase BjCHI1 inhibits growth of fungal phytopathogens and agglutinates Gram negative bacteria. *Journal of Experimental Botany*, 59: 3475–3484.

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

Lacy GH and Lukezic L. 2004. Pathogenic prokaryotes. *In* Plant Pathology: Concepts and Laboratory Exercises. (Trigiano RN, Windham MT and Windham AS Eds): CRC Press LLC, USA, pp 41-52.

Leppla N, Momol T, Nesheim N and Dusky J. 2004. Plant, Animal and Human Protection, FAS 2 Focus Area University of Florida / IFAS.

Mahadevan A and Sridhar R.1982. Methods in Physiological Plant Pathology, 2nd edition, Sivakami Publications, Madras.

Mauch F, Mauch-Mani B and Boller T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and ß-1,3- glucanase. *Plant Physiol.* 88:936-942.

Pfund C, Tans-Kersten J, Dunning FM, Alonso J, Ecker CA and Bent AF. 2004. Flagellin is not a major defense elicitor in Ralstonia solanacearum cells or extracts applied to Arabidopsis thaliana. *MPMI* 17: 696-706.

Schönfeld J, Heuer H, van Elsas JD and Smalla K. 2003. Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of *fliC* fragments. *Applied and Environmental Microbiology*, 69: 7248-7256.

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

Wessels JGH and Sietsma JH. 1981. Fungal Cell Walls: a survey in Plant Carbohydrates II. Extracellular Carbohydrates. *In*: Encyclopedia of Plant Physiology (Tanner W and Loewuse FA eds) Springer Verlag, Barlin, Germany, pp 352-394.