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).

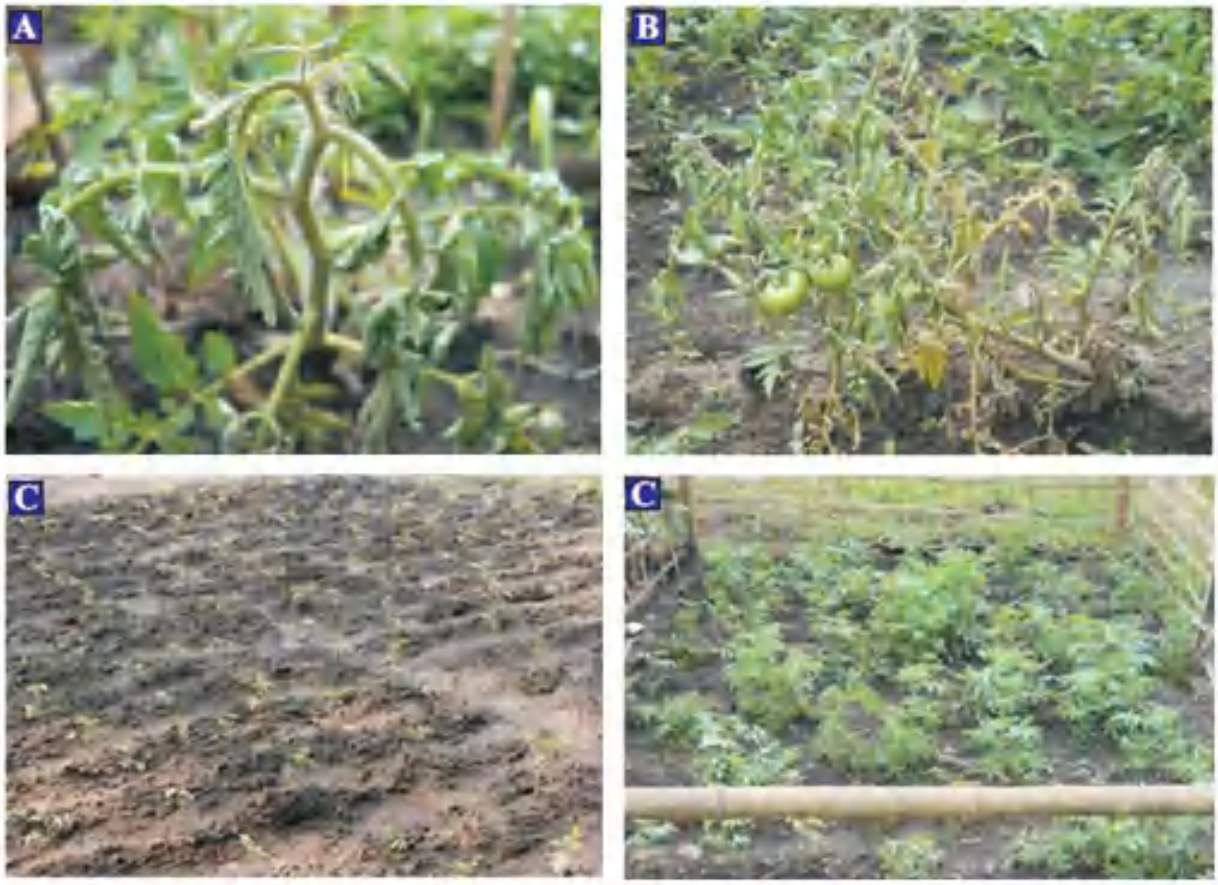


Fig 4.1: Infected plant in farmers field. a) Sonapur - Cooch Behar b) Haldibari - Cooch Behar c) Durgapur-Raiganj d) Ghokshadanga - Cooch Behar

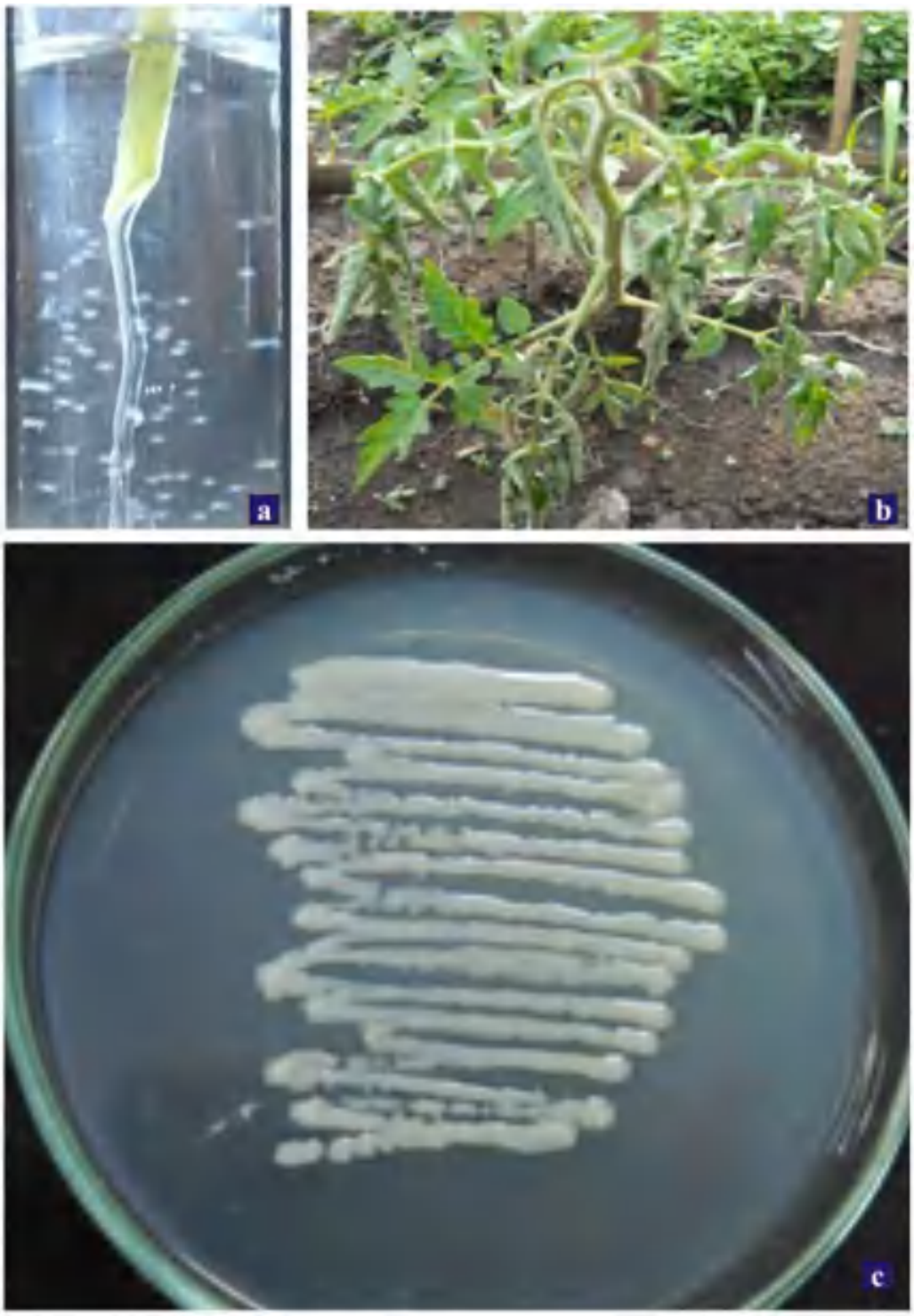


Fig. 4.2 : Isolation of bacteria from bacterial ooze. a) Collection of bacterial ooze in sterile distilled water b) Intected tomato plant from where ooze was collected . c) Pure culture isolation from bacterial Ooze.

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.

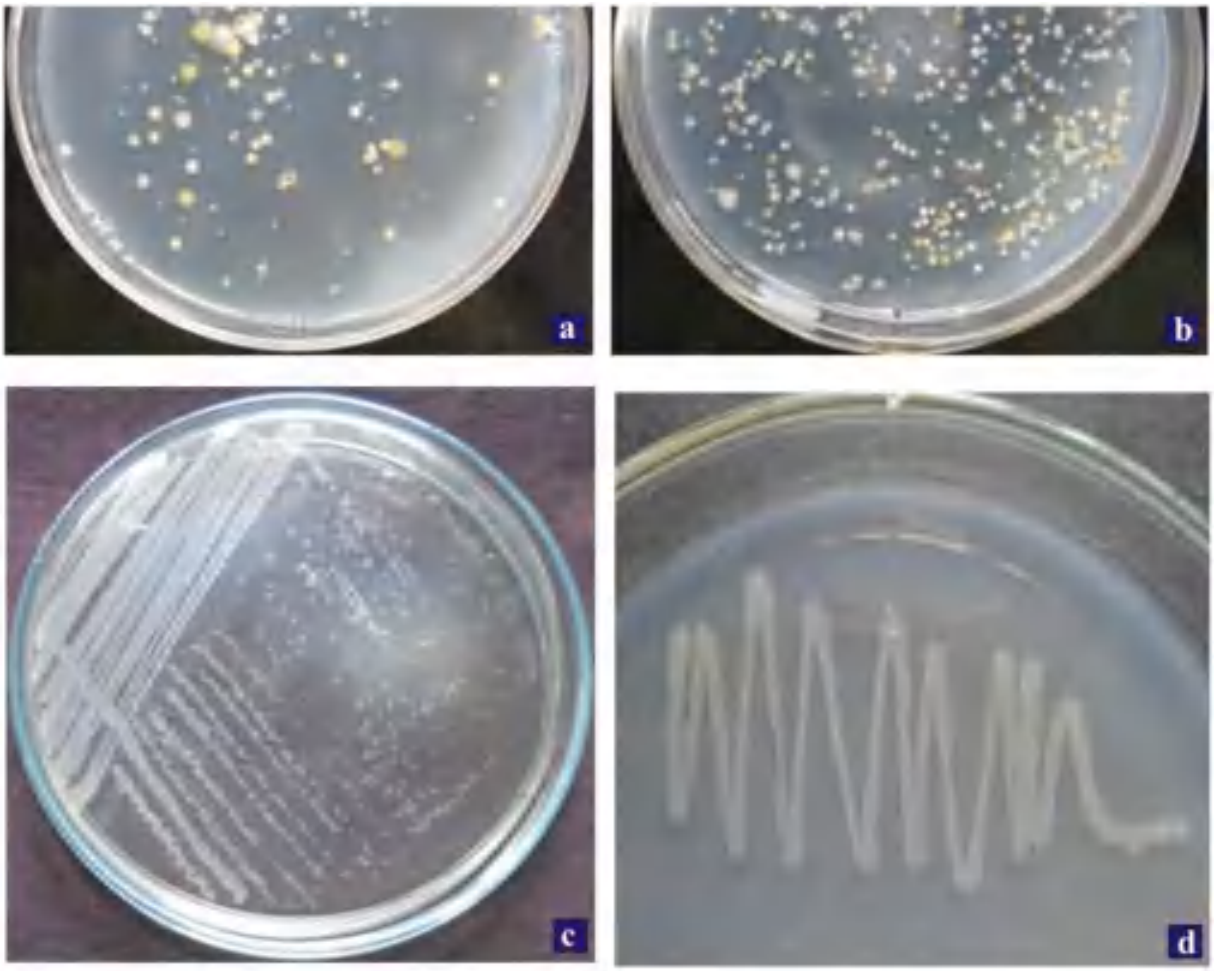


Fig. 4.3 : Isolation of bacteria by serial dilution and streaking. a) 10-4 Concentration plate b) 10-3 Concentration plate c) Single colony streak d) Pure culture isolation.

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).

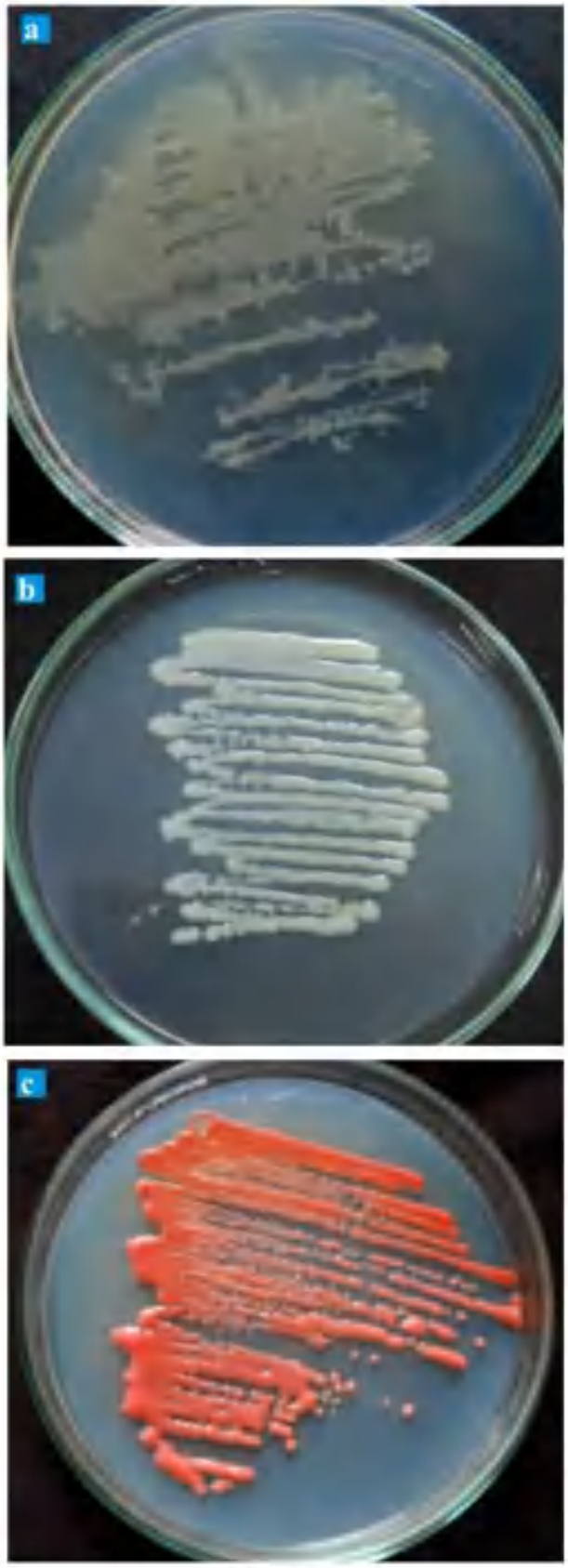


Fig.4.4: Growth of 3 different Ralstonia solanacearum (RSG01, RSG02 and RSG03) in CPG medium a) RSG01 b) RSG02 c) RSG03

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.

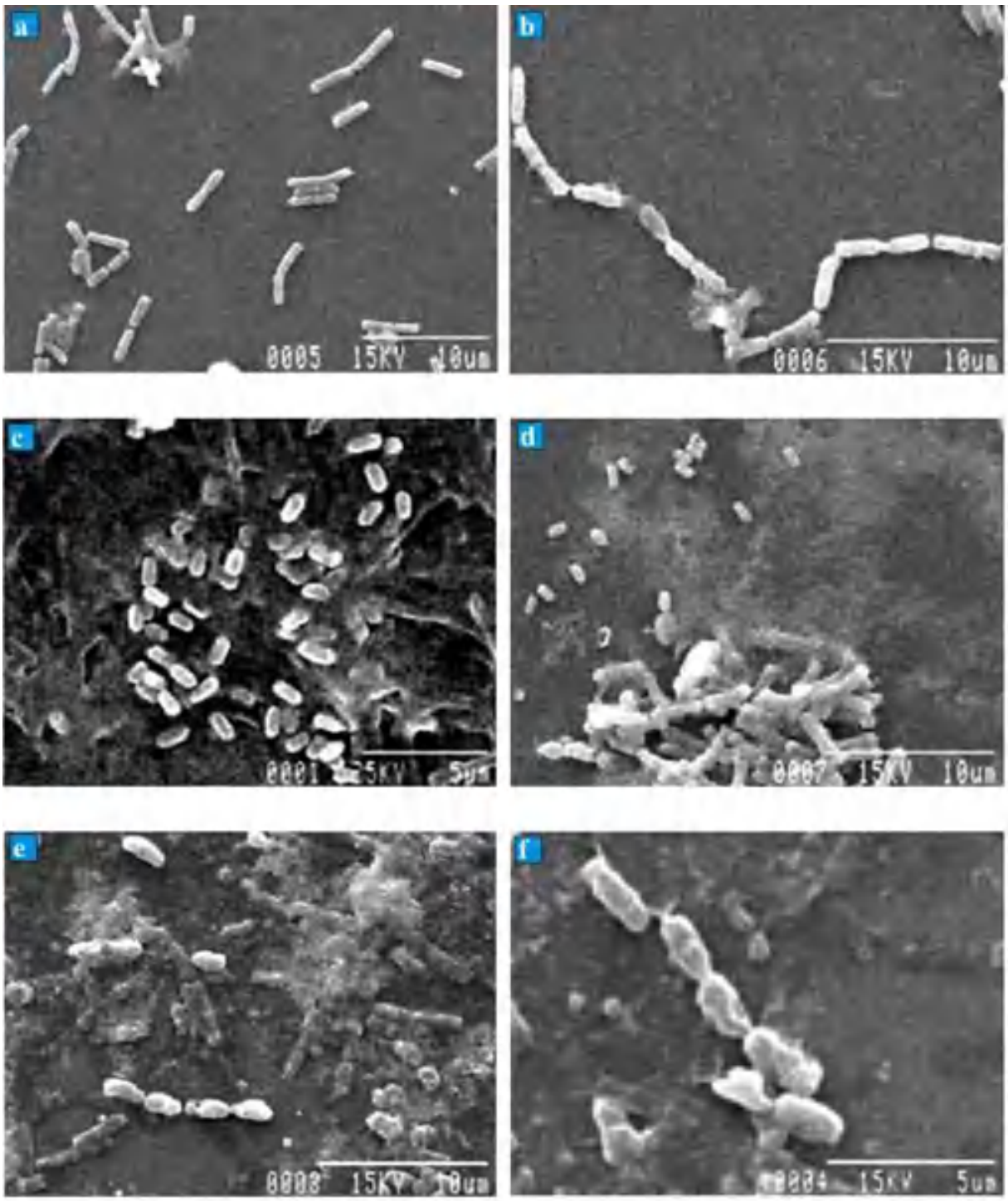


Fig. 4.5: Scanning electron micro photographs of 3 virulent bacteria RSG01, RSG02 and RSG03. a) & b) RSG01 isolate of (*Ralstonia solanacearum*) c) & d) RSG02 isolate of (*Ralstonia solanacearum*), e) & f) RSG03 isolate of (*Ralstonia solanacearum*),

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

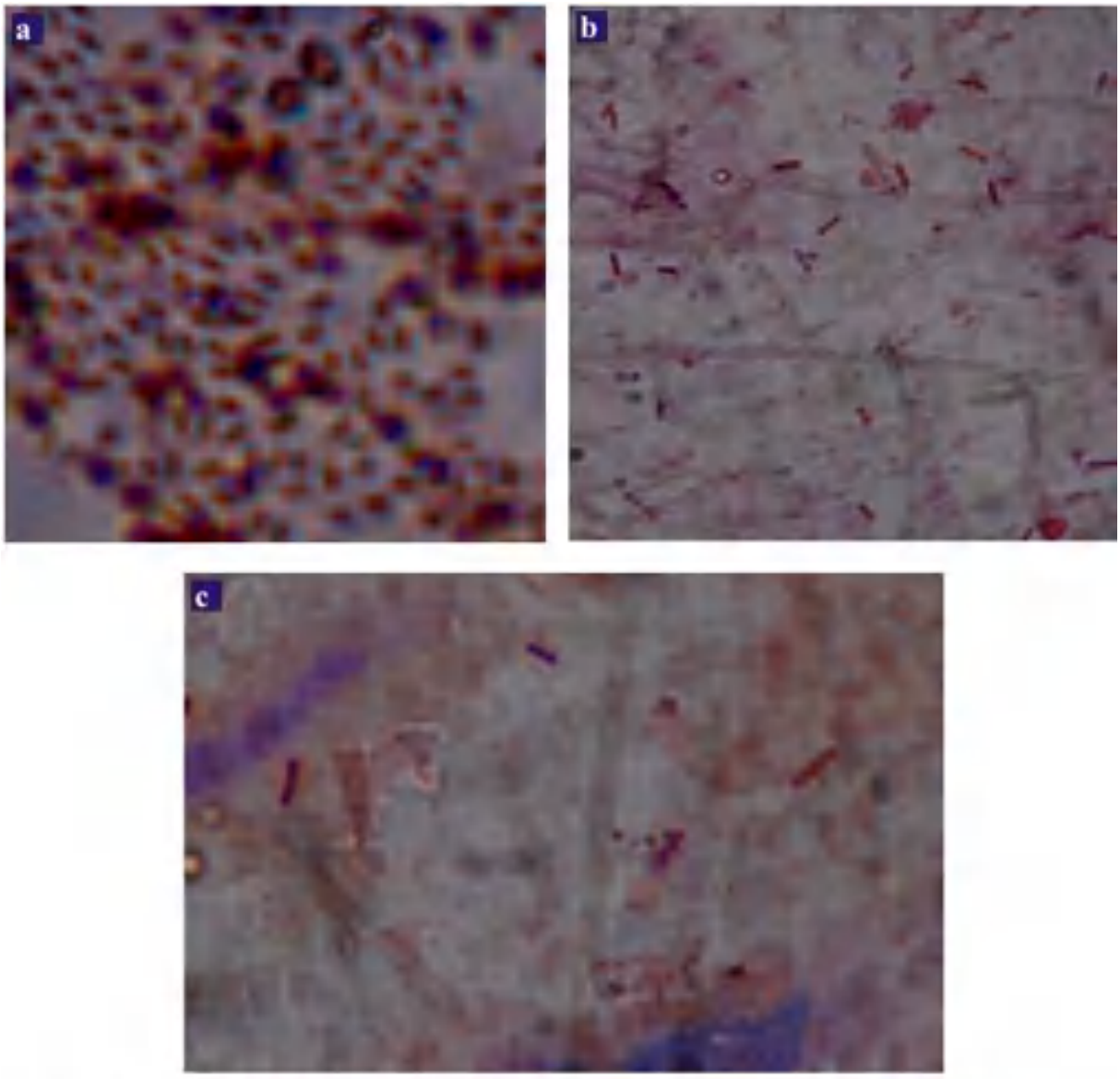


Fig 4.6: Morphological appearance of 3 verulent bacteria (*Ralstonia solanacearum*) a) Gram stained bacteria RSG01 b) Gram stained bacteria RSG02 c) Gram stained bacterial RSG03

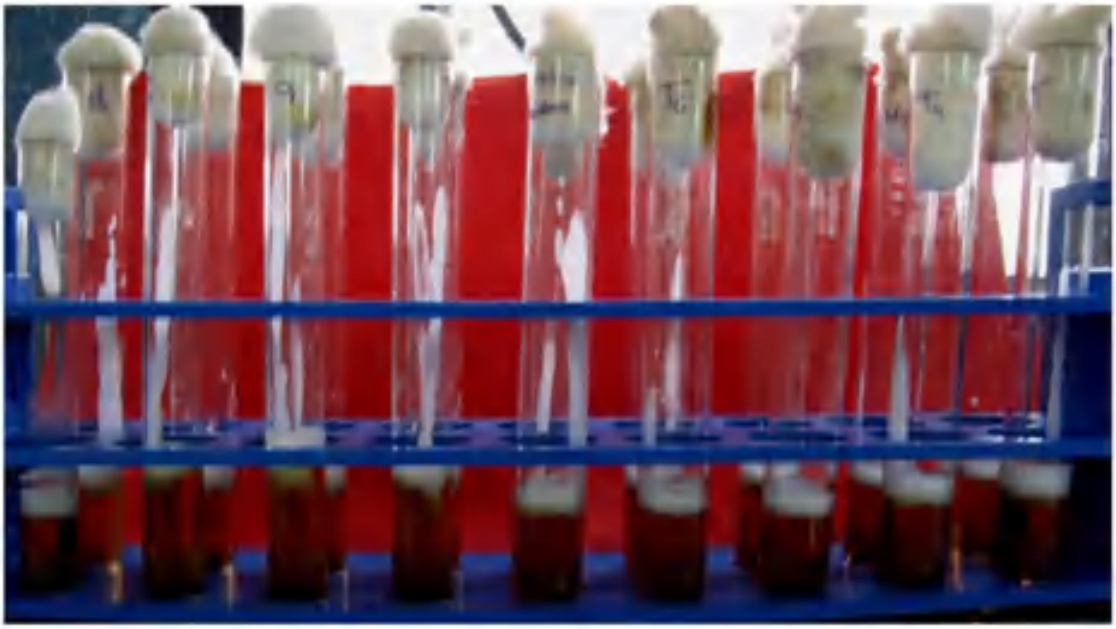


Fig 4.7: Anaerobic growth pattern test of the isolated cultures of Ralstonia solanacearum.

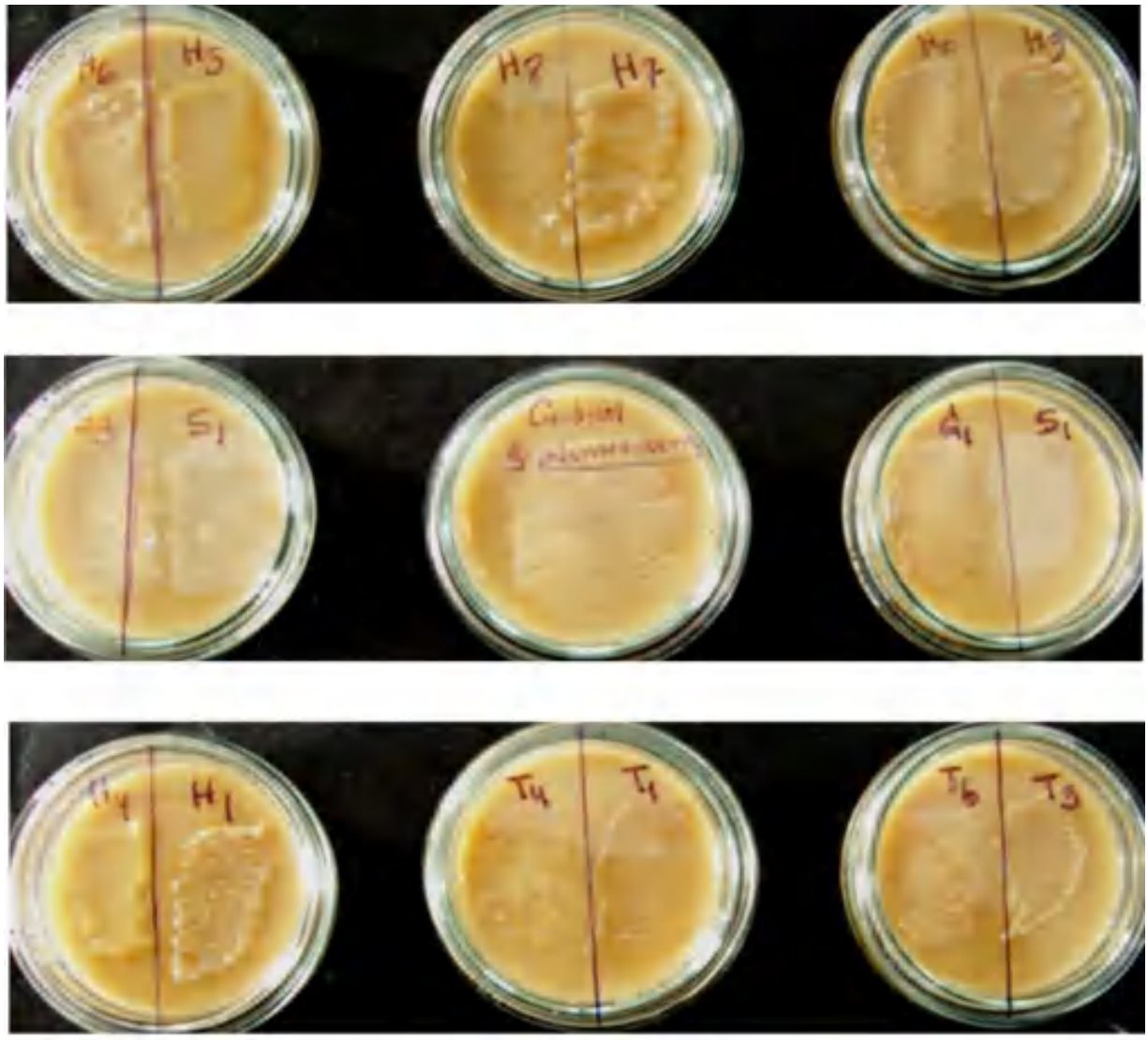


Fig. 4.8: Growth of 16 different cultures on YDC medium

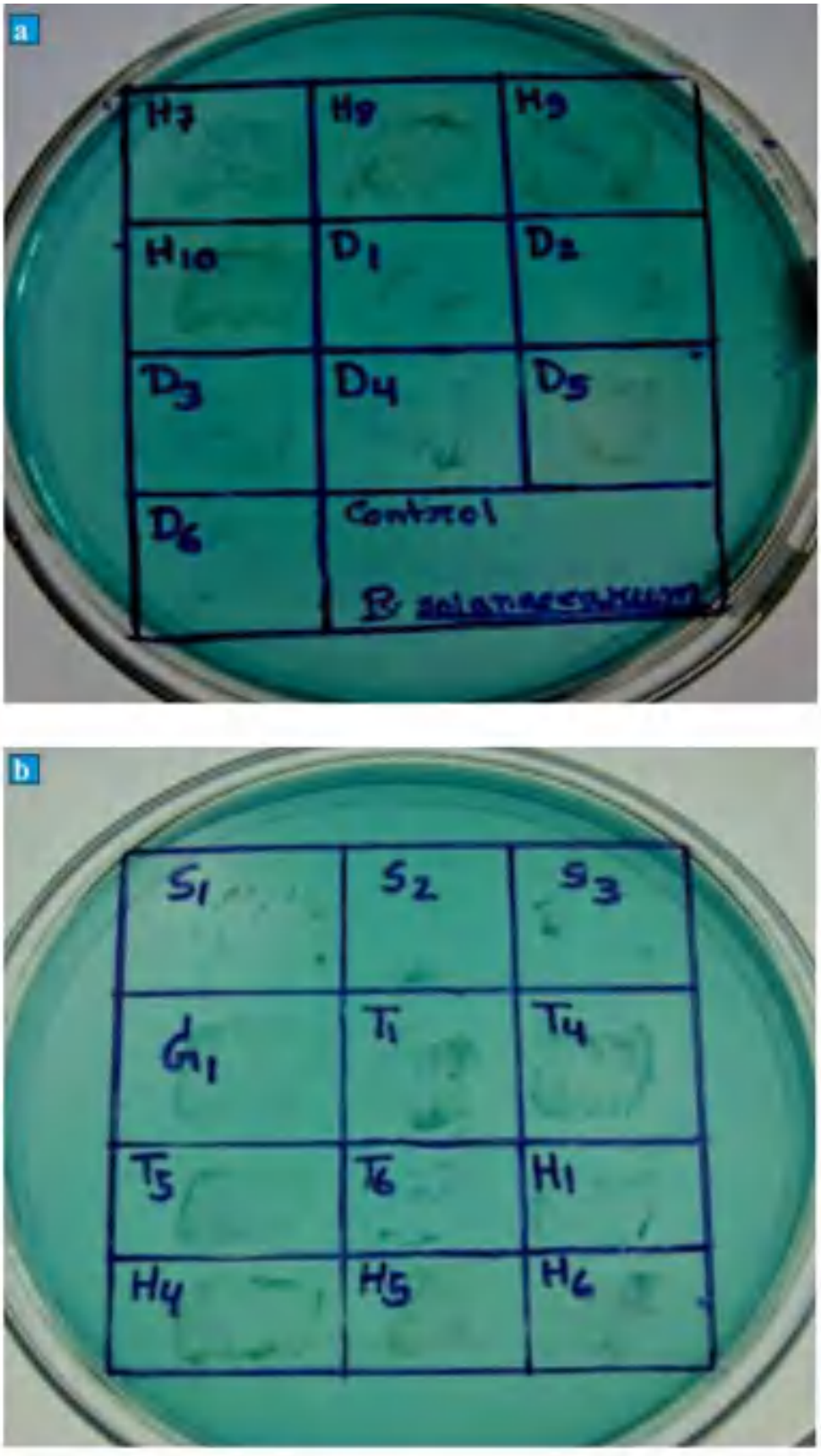


Fig. 4.9: a) & b) Growth of different Ralstonia solanacearum in D1M medium.

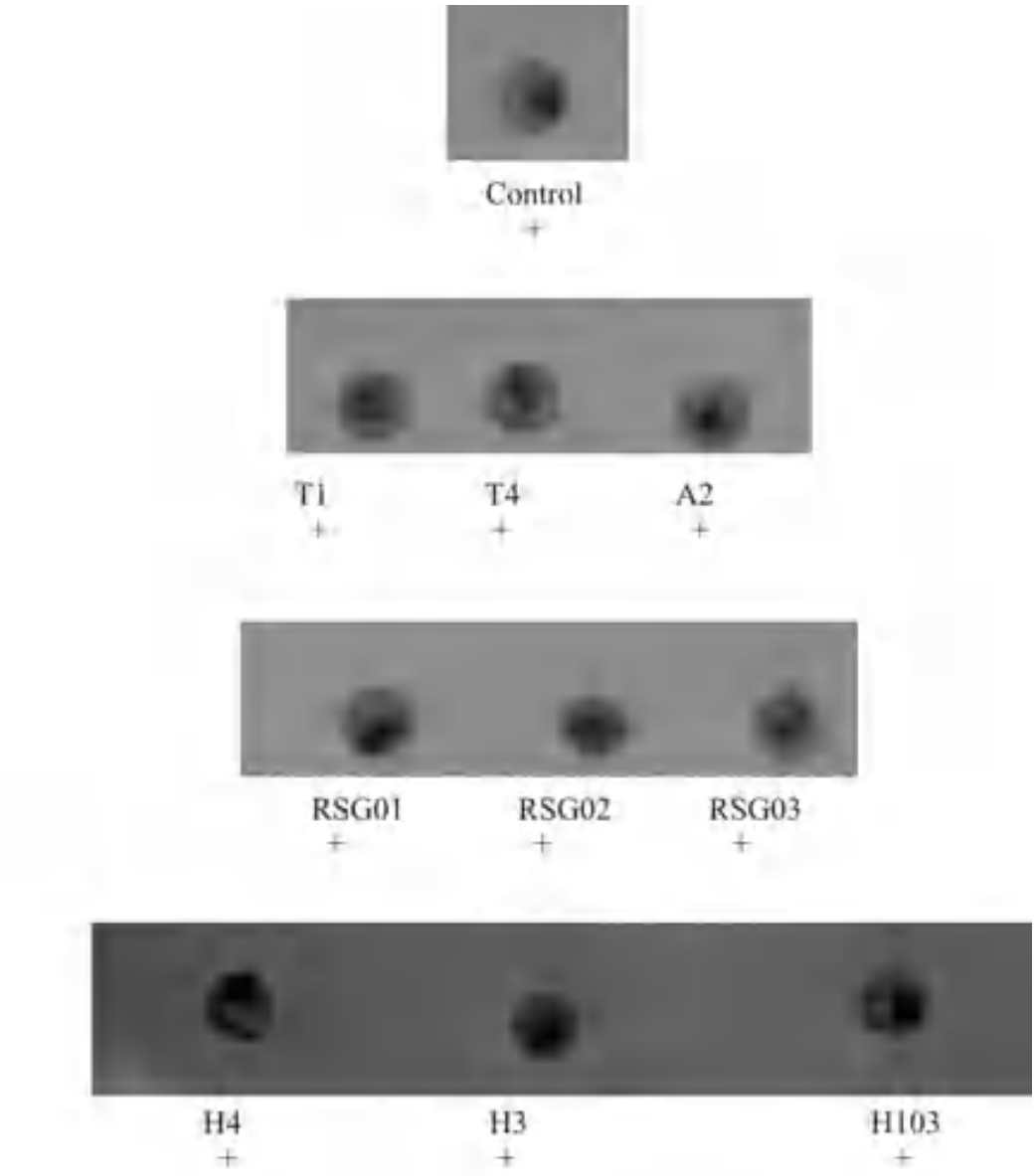


Fig 4.10: Oxidase test of some isolated bacteria

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

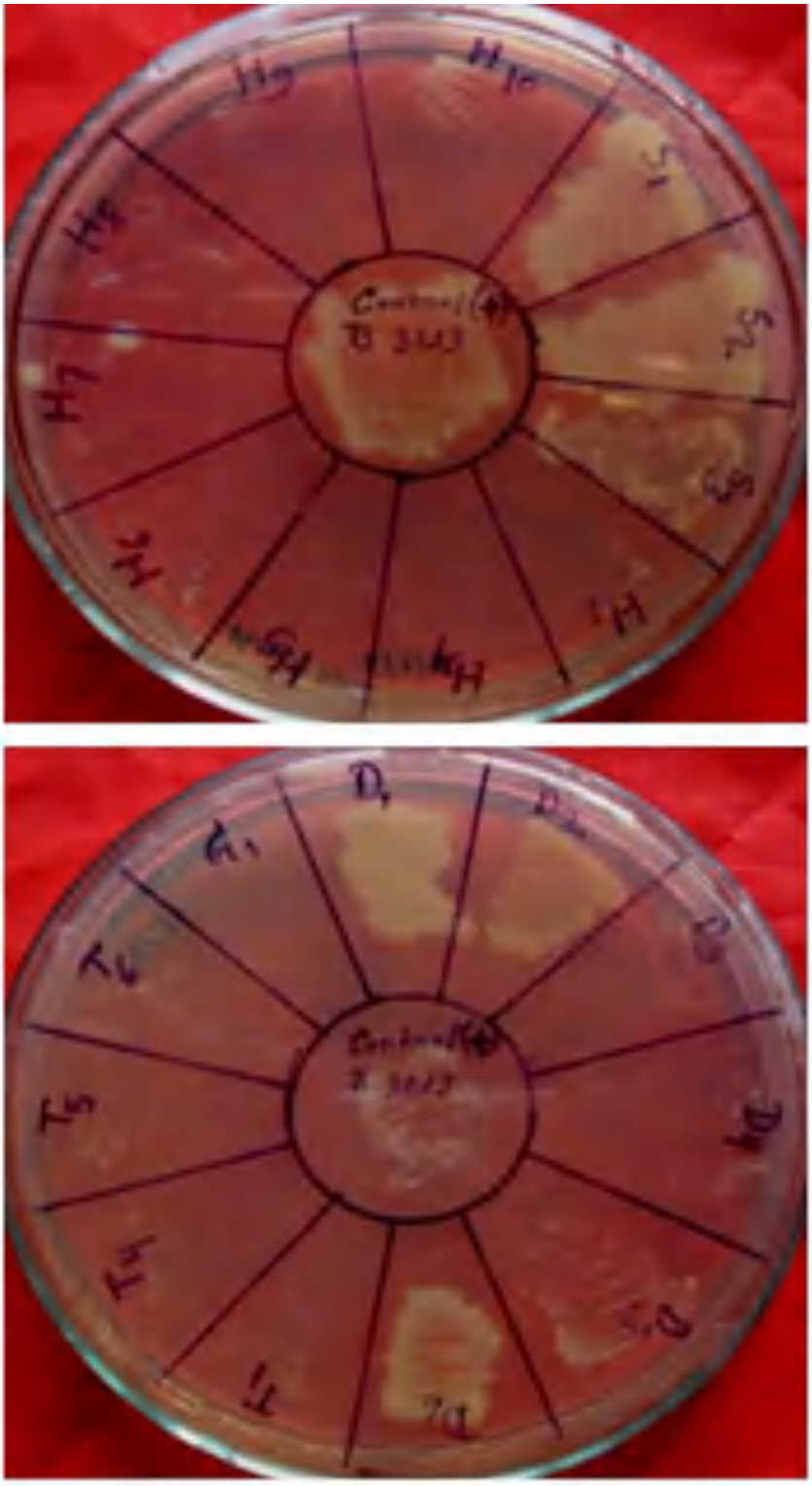


Fig. 4.11: Growth of different isolated and purified cultures on PSA medium.

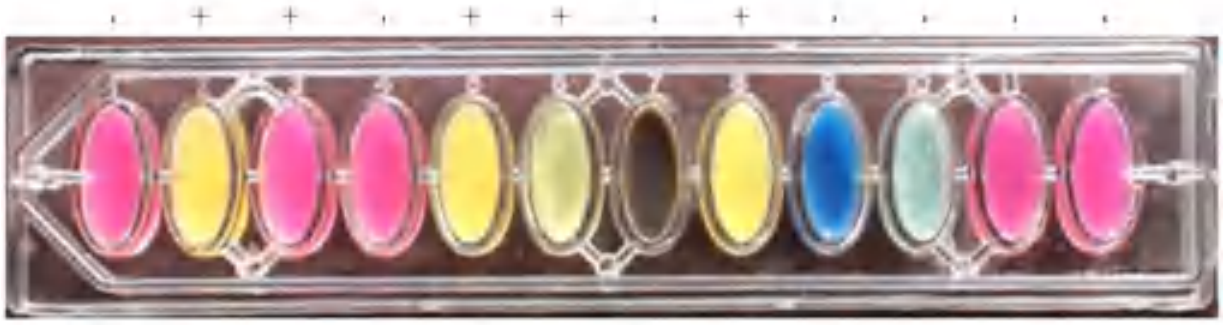
red test, Voges Proskauer's test, Citrate utilization test, Glucose utilization test, Adonitol utilization test, Arabinose utilization test, Lactose utilization test, Sorbitol utilization test, Manitol utilization test, Rhamnose utilization test and Sucrose utilization test. The results have been presented 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)

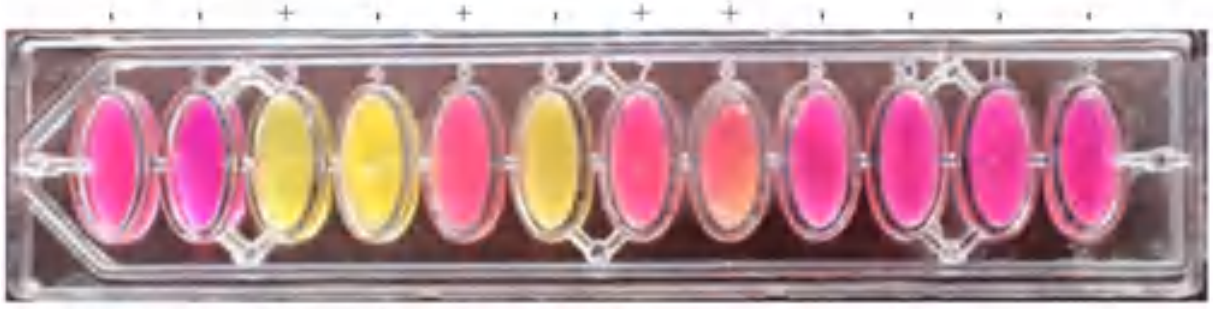
Fig. 4.12: Carbohydrate utilization test of RSG01 isolate of *Ralstonia solanacearum*

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**).

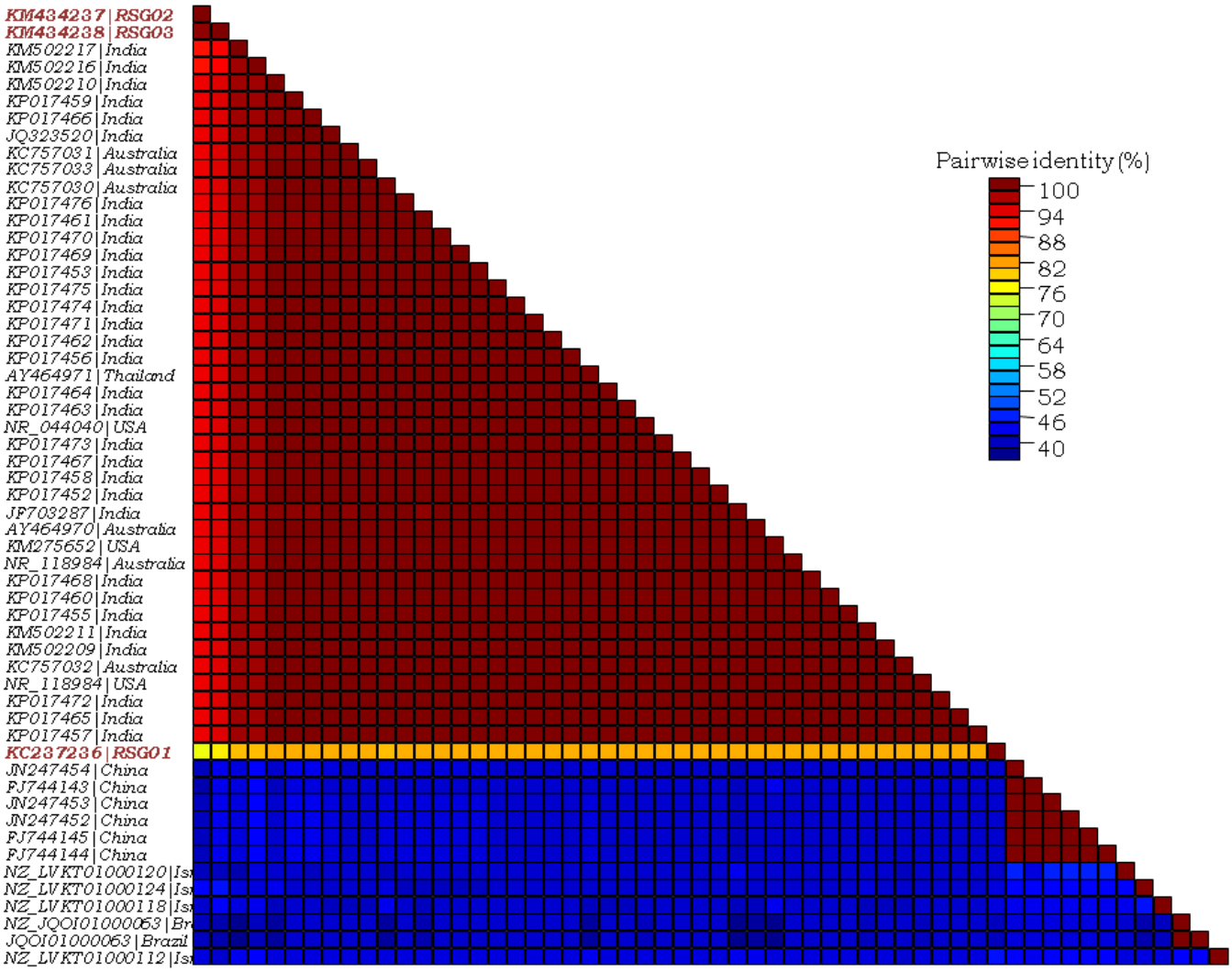


Fig. 4.15: Nucleotide sequence identity matrix of *three Ralstonia solanacearum isolates of the present study and other Ralstonia solanacearum of GenBank following 16s rRNA sequence analysis. Identity percentages are indicated on the right side corner of the matrix.*

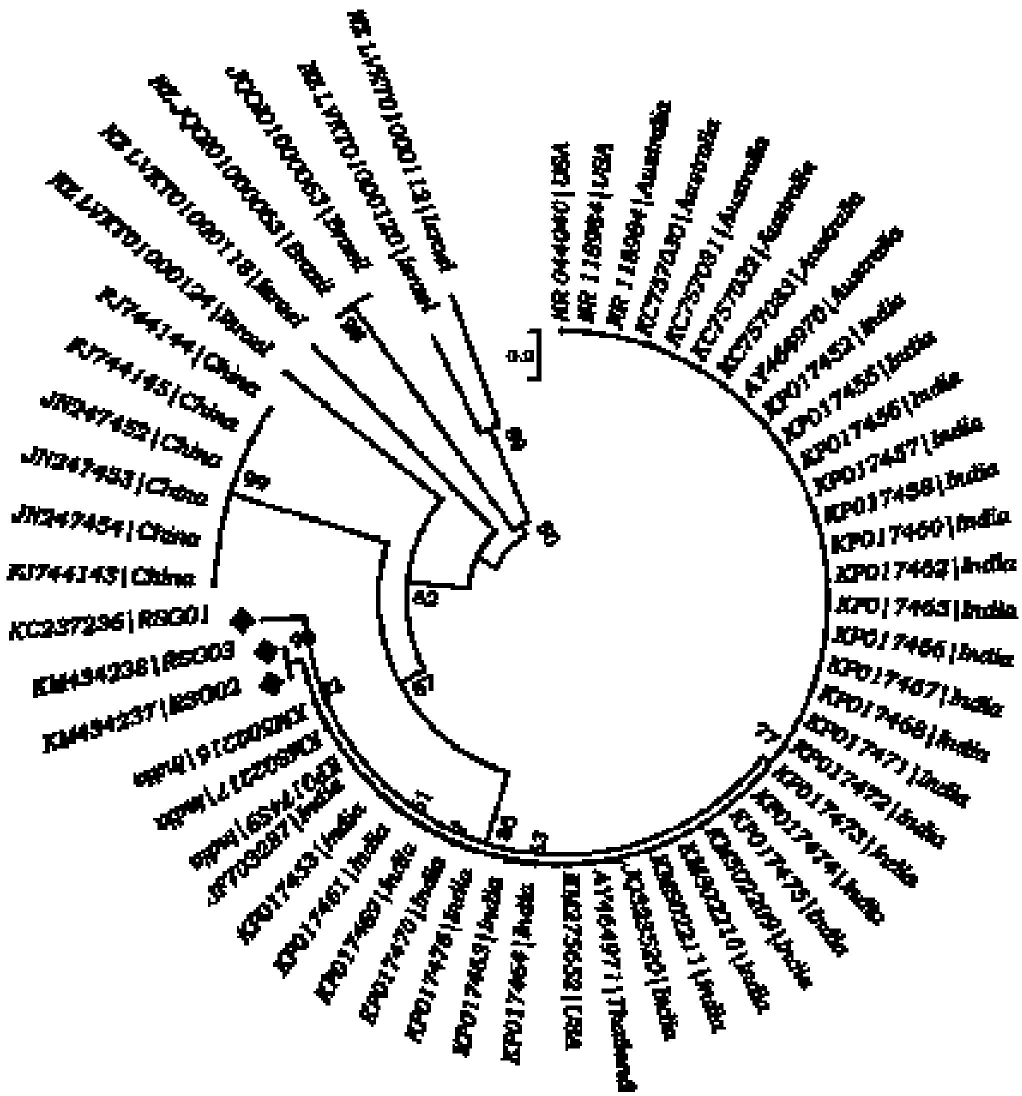


Fig. 4.16: Phylogenetic tree generated by neighbour joining of different *Ralstonia solanacearum. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates) and are indicated if greater than 50. GenBank accession numbers of the bacteria have been indicated at the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

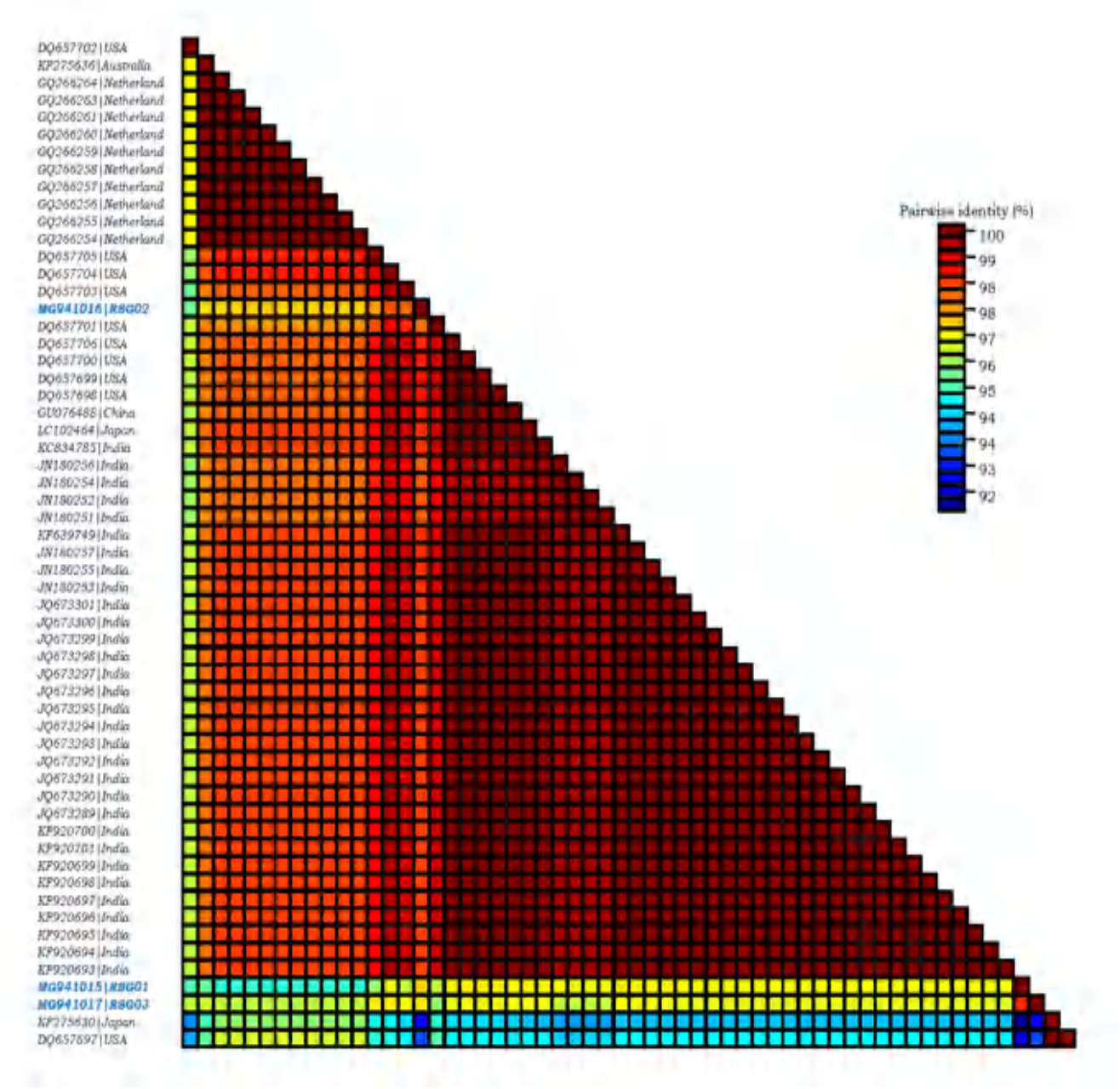


Fig. 4.17: Nucleotide sequence identity matrix of *three Ralstonia solanacearum isolates of the present study and other Ralstonia solanacearum of GenBank based on fliC gene analysis. Identity percentages are indicated on the right side corner of the matrix.*

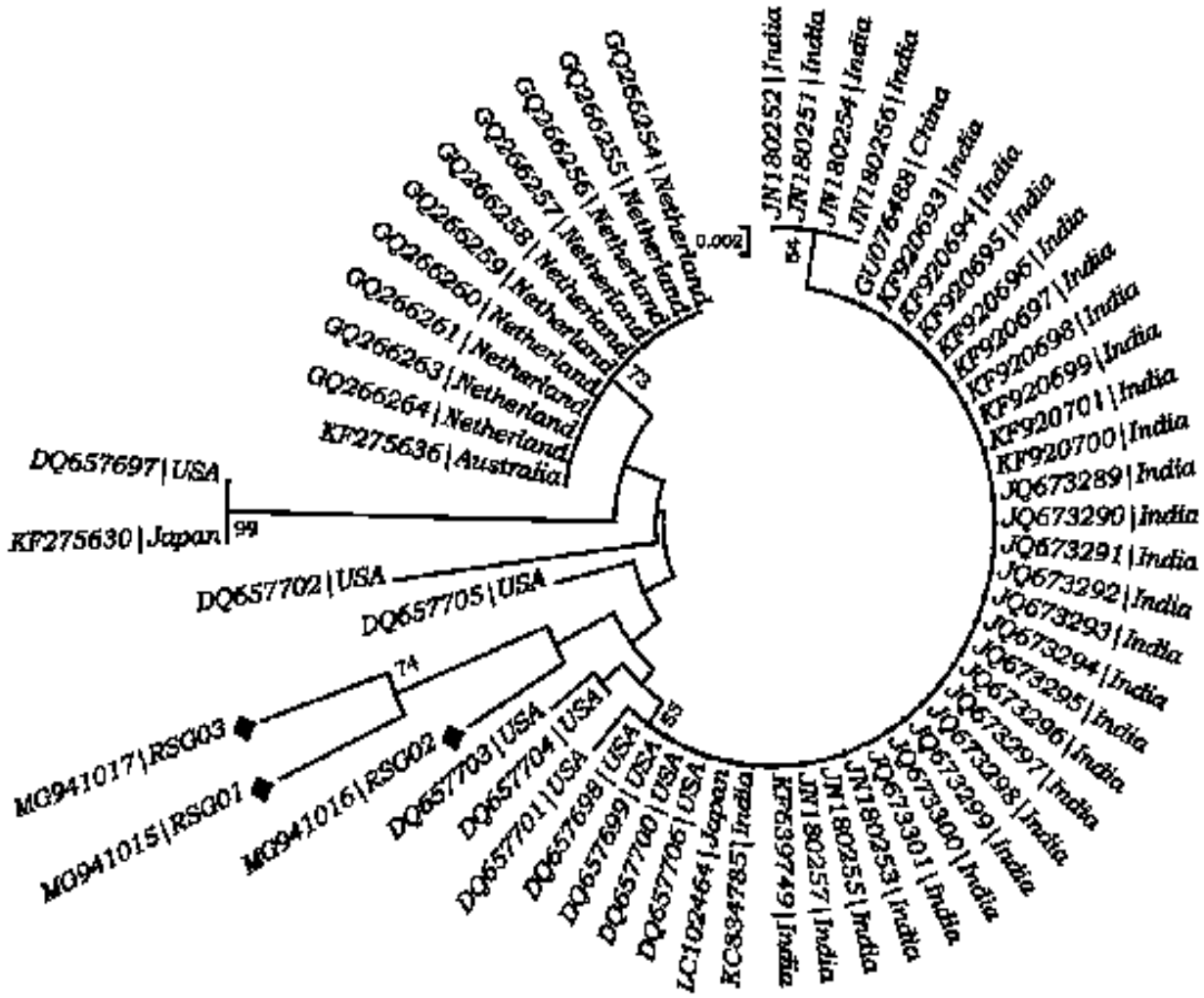


Fig. 4.18: Phylogenetic tree generated by neighbour joining of different *Ralstonia solanacearum based on fliC gene. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates) and are indicated if greater than 50. GenBank accession numbers of the bacteria have been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

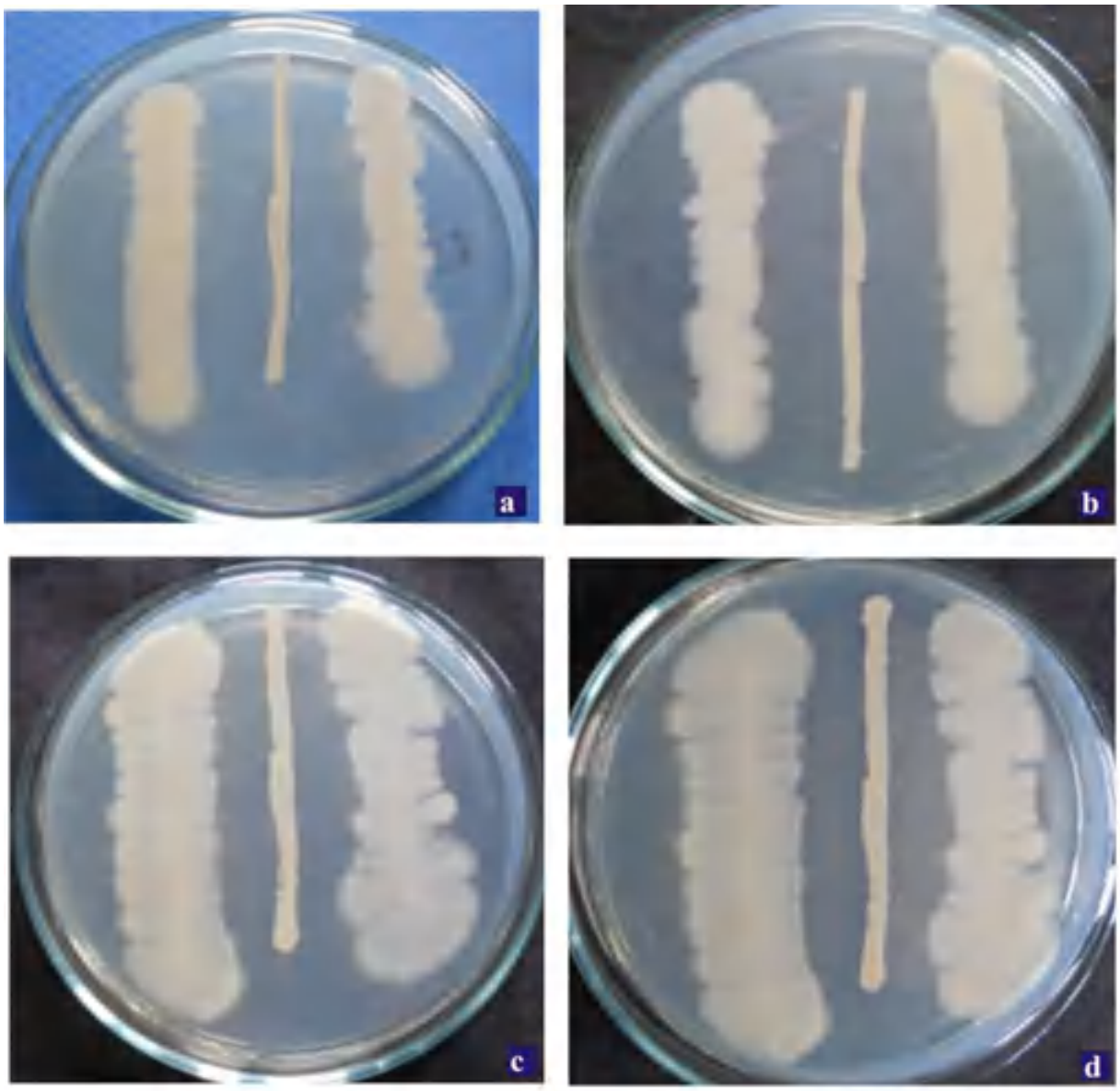


Fig. 4.19: Duel culture test of Ralstonia solanaceurm strain RSG01 against antagonistic bacteria Bacillus sp. Isolated from North Bengal.
a) Observation after 12 hr. b) observation 24 hr. c) observation after 48 hr. d) observation after 72hr.

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

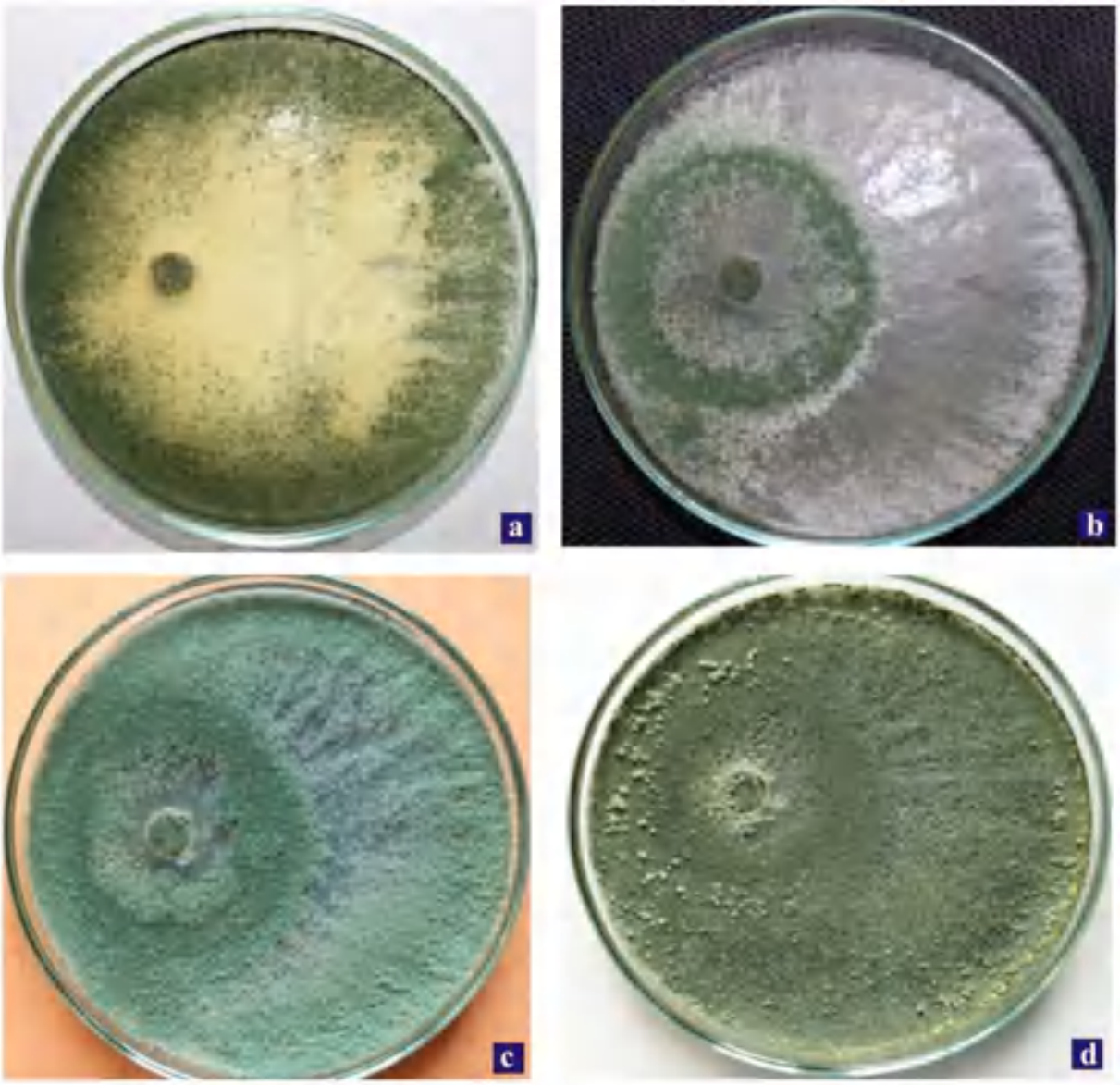


Fig. 4.21: In vitro dual culture test of Ralstonia solanacearum and Tricoderma harzianum a) Observation after 48hr. b) Observation 72 hr. c) Observation 96hr d) Observation after 120 hr.

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.

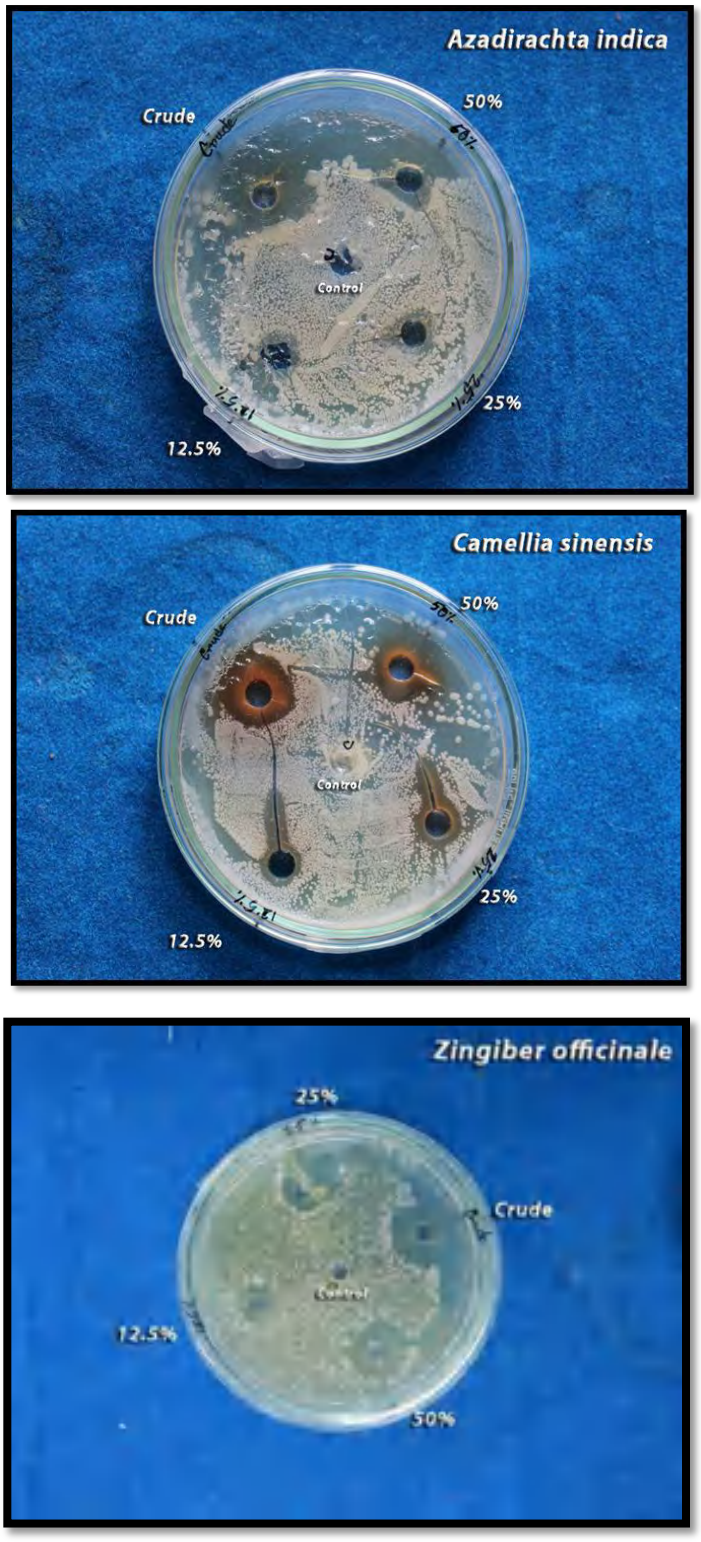


Fig. 4.22 Inhibition of growth of *Ralstonia solanacearum* by *Azadirechita indica*, *Camellia sinensis* and *Zingiber officinale*

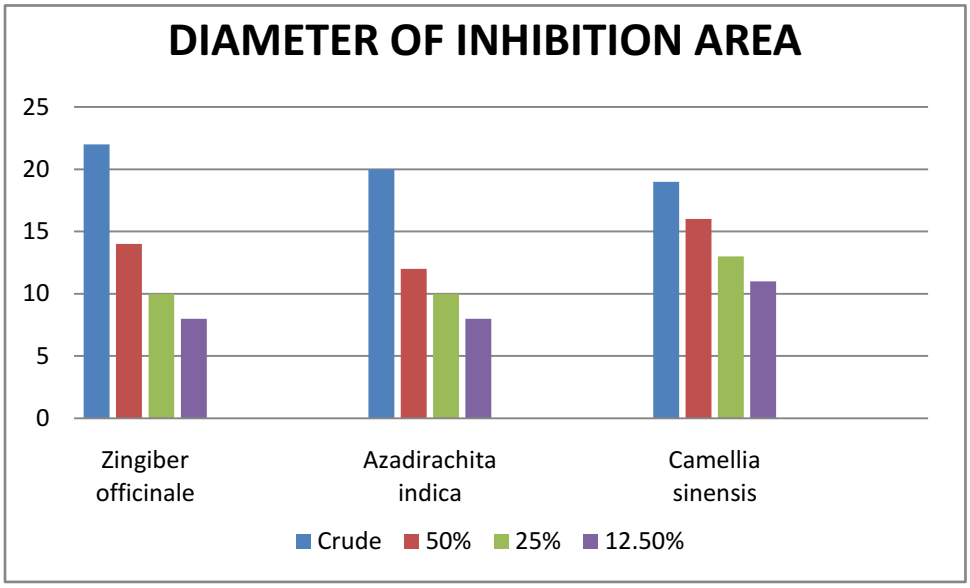


Fig. 4.23. Graphical representation of inhibition of growth of 3 selected botanicals in four different concentrations

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