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

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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^{TM} 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.

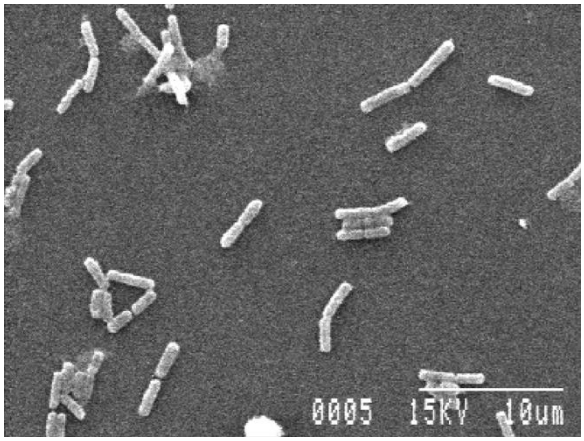

Figure 2. Scanning Electron Microscopic image of RSG01.

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).

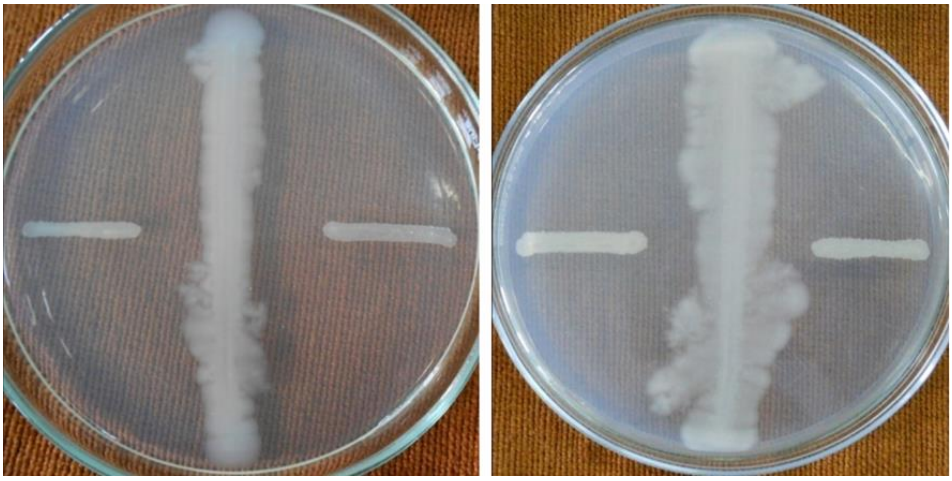

Fig-3: a) Dual culture of *Bacillus cereus* and *Ralstonia solanacearum* isolate RSG01 and b) Dual culture of *Bacillus cereus* and *Ralstonia solanacearum* isolate RSG02 .

***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.

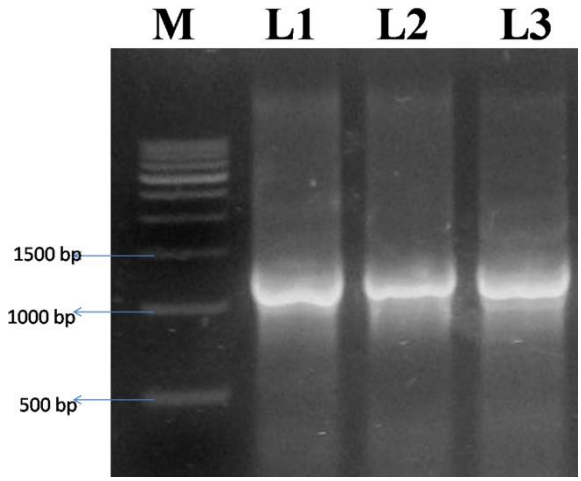

Figure 4. Expected amplicons (raised through PCR using suitable 16s rRNA primer pairs) of *R. solanacearum* isolates on agarose gel. M= 500bp ladder; L1, L2, L3= RSG01, RSG02, RSG03 isolates respectively.

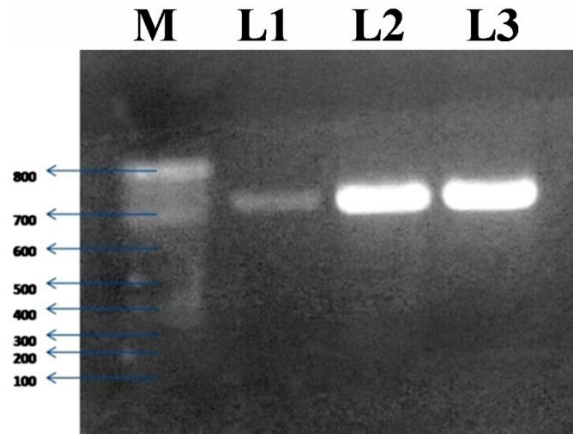

Figure 5. Expected amplicons (raised through PCR using suitable 16s rRNA primer pairs) of *Bacillus cereus* isolate on agarose gel. M= 100bp DNA ladder; L1, L2 and L3= HS01 isolate

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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