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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. solonacearum* 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 (Goellner 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.

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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.

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

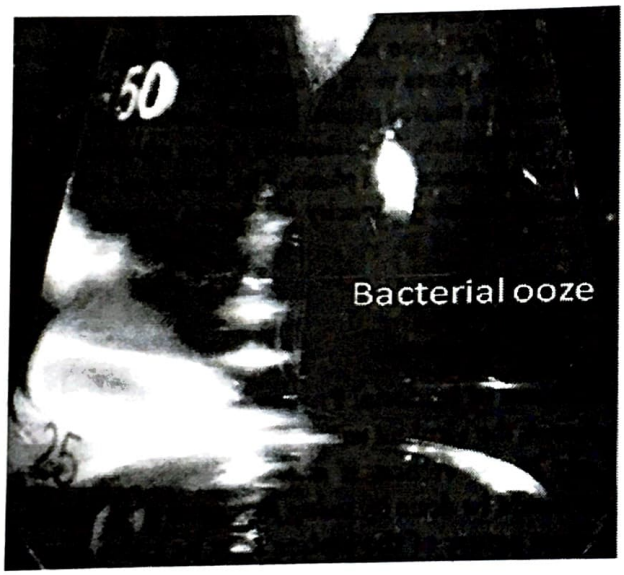

Figure 1. Bacterial ooze coming out from the cut end of stem of infected tomato plants.

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_6) 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_4	++
3	Haldibari (Cooch Behar)	T_5	+
4	Haldibari (Cooch Behar)	T_6	+++++
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	Durgapur (Uttar Dinajpur)	A_1	+
12	Durgapur (Uttar Dinajpur)	A_2	++
13	Durgapur (Uttar Dinajpur)	A_3	+
14	Durgapur (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	Ghoksadanga (Cooch Behar)	S_1	+
25	Ghoksadanga (Cooch Behar)	S_2	+
26	Ghoksadanga (Cooch Behar)	S_3	++

*Names given in parentheses 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 S_3 and D_5) could produce pigment on YDC medium and the other 24 bacteria were unable to produce pigment. Only two bacteria (isolate D_4 and H_5) could grow at 40° C and above. But, no bacteria could grow at 4°C. One bacterium (isolate D_4) could grow on D1M medium. Out of the bacteria tested three bacterial isolates (isolate H_4, H_5 and A_3) 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 *Ralsonia 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.

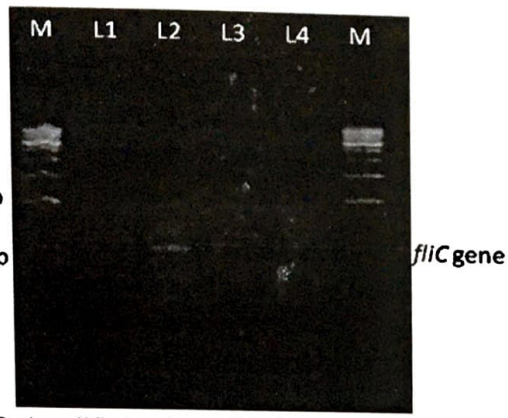

Figure 2. Amplified prduct of *fliC* gene of the virulent isolate T_6 on agarose gel after electrophoresis.

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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