Abstract

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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