Chapter-5

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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