CHAPTER 2

ISOLATION & CHARACTERIZATION OF *R. SOLANACEARUM* F1C1 ANTAGONISTIC BACTERIA FROM TOMATO SEEDLINGS AND STUDY THEIR EFFECT IN CONTROL OF BACTERIAL WILT IN TOMATO SEEDLINGS

2.1 Abstract

Plants harbor diverse microbial populations and the role of the different plant-associated bacteria and their interaction with the host has been an important topic of research for scientists. Endophytes are plant-associated microorganisms that reside in the internal tissues of the plant without exhibiting any visible symptoms. The role of endophytes in plant health has been elucidated in many plant-microbe interaction studies. It is found that several endophytes can exhibit antagonistic activity against plant pathogens and possibly plant uses these endophytes as an arsenal to protect themselves from the pathogens. It is hypothesized that plants might carries them from generation to generation in order to fight against some pathogens. In this study, we isolated twenty-one bacterial endophytes from tomato seedlings grown under sterile and soil-free conditions. All the isolates were characterized by 16S rDNA sequencing, for twitching motility, and by cellulase assay. Among all the bacterial isolates, five were observed to inhibit the growth of a bacterial phytopathogen *Ralstonia solanacearum* F1C1 *in vitro*. Further, the ability of the antagonistic isolates in controlling the disease caused by F1C1 in tomato seedlings were analysed by different co-inoculation experiments. It was found out that all five bacteria were able to reduce the symptoms caused by F1C1 in tomato seedlings.

2.2 Introduction

Endophytes are defined as microorganisms that successfully colonize the internal tissue of plants without showing any symptoms[1, 2]. The association of endophytes with the host plants can be obligated or facultative [3]. Endophytes influence plants by colonizing internally and living inside the belowground (roots) or aboveground plant tissues (stem and leaves), forming the plant microbial endosphere [4]. Although plant-endophyte interaction has been studied by many researchers recently, many conceptual aspects related to the nature of endophytes, their role in plant health, mode and mechanisms of entry and localization, etc. are yet not clear. Host-associated microbes

can colonize the host horizontally via the environment; vertically from within the parent to the offspring, or by mixed modes [5]. Among others, bacterial endophytes are a part of major research interest in recent years. Bacterial endophytes are known to exhibit various activities in host plants including plant growth promotion, and seedling emergence, and also it has been reported by various researchers that bacterial endophytes provide resistance against plant pathogens and environmental stress [4, 6]. It has been reported that several bacterial endophytes reside in the same niche similar to plant pathogens, which might help them to be suitable bio-control agents [7]. Many of the bacterial endophytes get associated with the host plant during the different developmental stages of the host plants through the environment by different modes, but a detailed investigation is required to know the different mechanisms that bacteria used to get entry to the host [4]. It might be that plant chooses some of the endophytes and they become an integral part of the host and are carried from generation to generation to provide different benefits to the host. The bacterial endophytes are known to inhibit pathogens by the production of secondary metabolites or inhibitory chemicals, secreting different enzymes, inducing systemic resistance in the host, and competing for nutrition [2, 8]. Although the role of bacterial endophytes in plant health and against plant diseases are well reported [1,8,9,10] to have in-depth knowledge of the intricacies of interaction between plants, endophytes, pathogens, and the environment more research is required.

Several plant pathogens are known to colonize the endosphere of plants and then spread through the whole plant and cause diseases. *Ralstonia solanacearum* is one such bacterium that colonizes the internal vascular tissue of plants before spreading to the whole plant [11]. *R. solanacearum* is a Gram-negative, soil-borne bacterium which causes lethal bacterial wilt disease in more than 450 plant species from 54 different botanical families of monocot as well as dicot plants, which includes many important crops such as tomato, potato, brinjal, chili,etc [12]. *R. solanacearum* is considered as one of the most devastating phytopathogens due to its wide host range, aggressive nature of the disease, high genetic diversity, and adaptability in different environments [13, 14]. In addition to its genotypic variation and severity, this bacterium has an astounding capacity to survive in the soil for many years and forms latent infections within indigenous weeds, resulting in a big challenge in the eradication of this bacterium [14]. To control the disease crop rotation, use of pesticides/chemicals, plant breeding, field sanitation, and

use of tolerant cultivars have been practiced but all these approaches have been found not adequate to efficiently control the disease [15]. Albeit chemicals and bactericides are used, but those are not effective in controlling the disease. Moreover using chemicals can be toxic to the nearby environment, which may harm animals including humans. In addition, as the bacterium can reside in depth in the soil and colonizes the internal tissue of the host plant using chemicals will not be adequate. Using stable antagonistic microorganisms against the pathogen as biological control agent can be an alternative to mitigate the disease..Using microbes that have antagonistic activity against *R. solanacearum* as a bio-control agent against the pathogen can be an alternative approach to fight the disease. A range of recent studies has shown that plant-associated antagonistic bacteria havea potential bio-control effect against the *R. solanacearum* infection in laboratory and field conditions. [15-18].

R. solanacearum F1C1 strain was isolated from a wilted chili plant, collected from a nearby field in Tezpur, Assam, India[19]. Different methods were developed to study the pathogenicity of F1C1 in seedlings stages of tomato and brinjal in the author's laboratory [20-23]. The seedlings used in these studies are six to seven days old, freshly grown, and have two cotyledon leaves. The seedlings are germinated in a controlled environment to support gnotobiotic conditions and the pathogenicity assays are performed in hydroponic conditions. These assays are very easy, and less time-consuming as the whole process from seed germination to completion of pathogenicity assay takes around 15 to 20 days. Due to the soil-free nature-controlled environment, the interference of other microbes during the time of infection in these assays is negligible. During the development of these assays, we were fascinated to find out what kind of microbes, especially bacterial endophytes are present inside these seedlings and if they have any antagonistic activity against F1C1 or not.

In this study different endophytic bacteria were isolated from seven-day old tomato seedlings and each of them was tested for antagonistic activity against F1C1.Out of the 21 bacterial isolates 5 were found to have antagonistic activity against F1C1 in vitro. All the bacterial isolates were studied for twitching motility, cellulose assay, and were identified by 16S rDNA sequencing. All the 21 isolates were found to be non-pathogenic to the seedlings upon re-inoculation. The five antagonistic isolates were studied for bio-control potential using methods developed in the laboratory. Already few groups have

used these assays to study biological control study against *R. solanacearum* using different bacteria [24-26]. In our study, the bacterial isolates were isolated from tomato seedlings and were tested in tomato seedlings for their bio-control potential against F1C1. We found that all the five antagonistic bacteria were able to reduce the disease in the tomato seedlings individually also as consortia; as well the symptom appearance was delayed.

2.3 Materials and Methods

2.3.1 Bacterial strains, growth media and culture conditions

Bacterial strains used in this study have been enlisted in Table1. The wild type *R. solanacearum* F1C1 [19] and the bacteria isolated from tomato seedlings were grown on BG medium supplemented with glucose ($5gL^{-1}$ at final concentration). The composition of BG medium is as follows: 1% peptone; 0.1% yeast extract; 0.1% Casamino acid; 1.5% agar is added for solid medium. F1C1 strain and the isolated bacteria were grown at 28 ºC. The E. coli MG1655 was grown on LB medium and agar was added for solid medium. The MG1655 strain was grown at 37 ºC.

Table 2.1: Bacterial strains used in this study

Sl No.	Strain	Characteristics	Reference/Source
1	F1C1	Wild type *R. solanacearum* strain (phylotype I), isolated from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India	[19]
2	MG1655	Wild type *Escherichia coli*	Lab collection

2.3.2 Germination of tomato seedlings

The tomato cultivar Durga Pusa Ruby was recruited for bacterial endophyte isolation, bio-control assay, virulence test etc. The seeds were pre-soaked in sterile distilled water and were kept overnight. Thenthe seeds were disinfected by washing in 70% ethanol for 1-2 minutes, followed by washing with sterile distilled water for 2-3 times and then soaked in sterile distilled water and for overnight. Next day the seeds were sowed on

wet seedbed comprising of sterile absorbent cotton and sterile tissue paper. The seedbed was transferred in growth chamber and the seeds were allowed to germinate for seven days or till it become ready for bacterial inoculation. Sterile distilled water was sprinkled at the regular interval of time till 7 days to sustain the germination process and optimum conditions for germination were maintained 28 ºC temperatures, 80% Relative Humidity and a photoperiod of 12 h [20-23].

2.3.3 Isolation of tomato seedlings endophytic bacteria

For the isolation of endophytic bacteria, about 5gm of 7 days old two cotyledon leaf tomato seedlings were selected and subjected to surface sterilization. The seedlings were surface disinfected by stepwise washing with sterile distilled water,0.04 % Bavistin (w/v) for 2min, 0.04 % Mercury Chloride (w/v) for 30sec and 70 % ethanol for 1min; each step was followed by 2-3 times washing with sterile distilled H_2O [19].

The surface disinfected seedlings were crushed using a sterile mortar pestle. A homogenate was prepared by mixing the crushed seedling in 2 ml sterile distilled water. The resulting homogenate was serially diluted to 10^{-1}, 10^{-2}, 10^{-3} concentrations and 100 µl of each dilution was spread on BG agar plates in triplicates. Plates were incubated at 28°C for 4 days and observed intermittently for appearance of microbial colonies. Morphologically distinct colonies were selected and purecultures of selected colonies were obtained for further studies. For regular use, pure cultures were kept at 4 °C and for long term storage pure cultures were preserved at −80 °C in 25 % (v/v) glycerol.

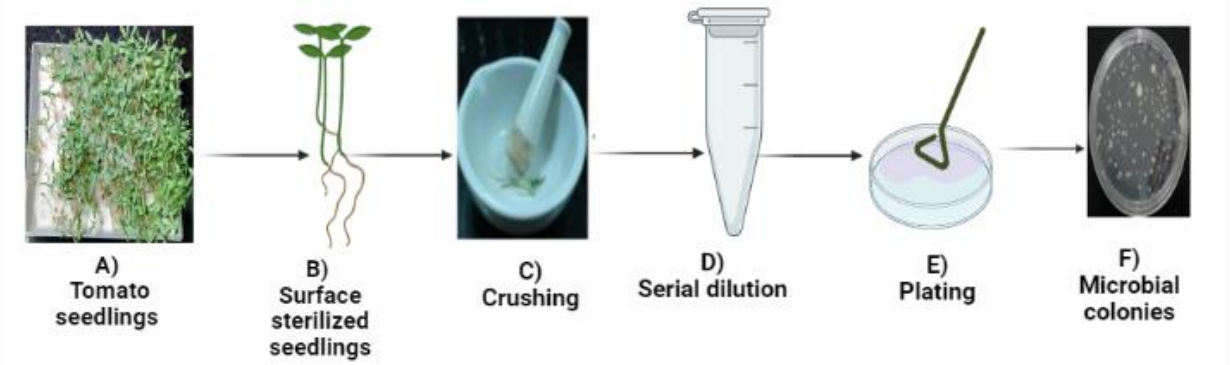

Fig 2.1: Schematic representation of steps involved in isolation of bacteria from tomato seedlings

2.3.4 Screening for *in vitro Ralstonia solanacearum* antagonistic activity

To evaluate the antagonistic activity of bacteria isolated from tomato seedlings two methods 1. Agar well diffusion and 2. Cross-streak methods were used.

1. Agar well diffusion method: For this assay*Ralstonia solanacearum* F1C1 strain was inoculated in 20mL BG liquid medium and allowed to grow at 28°C for 36 to 48 hrs ($1*10^9$cfu/ml), and all the endophytes were inoculated in 20ml BG liquid medium and allowed to grow till it reaches $1*10^9$cfu/ml. The F1C1 and all the endophytes cells were washed by centrifuging at 4000rpm for 15 min,then the supernatant were discarded and the cells were collected. Cells were washed twice with sterile distilled water and were re-suspend using sterile distilled water. BG agar plates were prepared in 90 mm diameter Petri dishes. Firstly, 100µl of F1C1 suspension was spread on the BG agar plates and kept for 30 minutes at 28°C, after that wells were prepared on centre of each plates using distal end of sterile 200 µl pipette tips. Then 30 µl of the endophytes suspension were individually poured into the wells of different plates to study their interaction against F1C1. Each endophyte was tested thrice. The plates were carefully kept at 28°C till 4 to 5 days, and were monitored regularly for any inhibition zone [27, 28]

2. Cross-Streak method: BG agar plates were prepared in 90mm Petri dish and first inoculated with F1C1 by a single streak of the inoculums in the center vertically. After that, the endophytes were streak perpendicular to F1C1. The plates were incubated at 28°C till4 to 5 days, and were frequently observed.

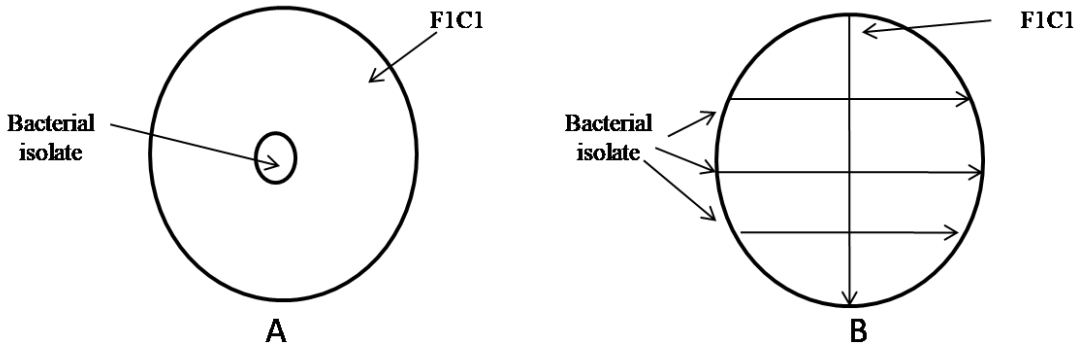

Fig 2.2: Schematic representation of *in vitro* assay. A) Agar well diffusion method: F1C1 was spread on the agar plate, and bacterial isolate was put on the well, B) Cross streak method: F1C1 streaked first on the plate, Bacterial isolate/s streaked perpendicular of F1C1 [29].

2.3.5 Interaction among the five *R. solanacearum* F1C1 antagonistic endophytes

To study if the antagonistic activity against F1C1 is specific or the endophytes interacts or inhibits each other agar well diffusion and Cross Streak methods were used as described earlier. In this study each one endophyte were tested against all the other endophytes individually.

For Agar well diffusion if one bacterium was spread on the agar plate, then the other one was inoculated in the well, and vice versa. So like this all the five antagonistic endophytes tested earlier were screened against each other. For Cross-Streak method one endophyte was streaked perpendicular to another one and like this all the five were tested against each other.

2.3.6 Effect of all the twenty one isolates on tomato seedlings

All the twenty onebacterial isolates were inoculated in tomato seedlings to find out if they have any deleterious effect on them. Although all the twenty one bacteria were isolated from healthy tomato seedlings, we thought it would be important to study their effect by again inoculating them in the seedlings. For the study, seven days old and two cotyledon leave stage tomato seedlings were recruited. All the isolates were freshly grown in BG broth medium overnight and similarly freshly grown F1C1 in BG broth medium was also taken for this study as positive control. All the bacterial cultures including F1C1 were subjected to centrifugation at 4000 rpm and 4 °C for 15 min and the cell pellets were resuspended with equal volume of water. Thereafter, the seven days old tomato seedlings were inoculated by the bacterial isolates individually by two different methods namely leaf clip inoculation method and root dip inoculation method.

In leaf clip method a small part of both the leaves were inoculated with the bacterial culture with the help of a pair of sterile surgical scissors. At first the pair of the scissor was dipped in the bacterial culture and then a small wound were made in both leaves and then the seedlings were maintained in1.5 ml sterile microfuge tube containing only sterile distilled water. The seedlings were observed for the occurrence of any symptom and regularly water of the tube was maintained. This way all the isolates and F1C1 was inoculated and checked for the pathogenicity. Each bacterium was inoculated in 20 seedlings. In one set the leaf of the seedlings were inoculated with only sterile distilled water [20, 22].

For root inoculation, roots of the seedlings were first dipped with the bacterial culture and kept in a sterile 1.5 ml microfuge tube, then after five minutes sterile distilled water was poured in to the tube. After the inoculation the seedlings were observed regularly for the presence of any symptom. The water level was maintained throughout the experiment. All the isolates were inoculated by the same method. Each bacterium was inoculated in 20 seedlings. In a set no bacterium was inoculated and kept as control [21, 23].

2.3.7Characterization of the bacterial isolates

2.3.7.1 Molecular Identification of the Isolates by 16S rDNA amplification and sequencing

i. 16S rDNA amplification

Molecular identifications of all the bacterial isolates were performed by amplification of 16S rDNA and sequencing of the amplified product. 16S rDNA sequences of all the isolated antagonistic bacteria were amplified by colony PCR with the help of universal primers [forward primer (27F) & reverse primer (1492.For colony PCR, single bacterial colony was first mixed in 5 µl of 200 mM NaOH and 95 µl of sterile distilled water in a sterile PCR tube and then it was kept for lysis at 95ºC for 10 minutes**.** The lysed products were used as template for PCR reaction. PCR reaction recipe for each 20µl volume reaction was: Dream Taq Buffer (10X) - 2.0 µl; 2 mM dNTPs - 1.2 µl; DMSO - 0.6µl; forward primer (10 µM) - 1.0 µl; reverse primer (10 µM) - 1.0 µl; template DNA - 1.0 µl; Dream-Taq DNA polymerase (5U/µl) - 0.2 µl; nuclease-free water - 13µl. Cycling conditions used for PCR were: initial denaturation at 95°C - 5 min, annealing at 60°C – 30 sec, extension at 72°C – 1.30 min, final extension at 72°C – 10 min. Amplified PCR products were visualize on 0.8% agarose gel (1X TAE, 70V, 200 mA, and 45 min) and the amplified products were first observed in a Gel-Doc system (E-gel imager, Thermo-Fisher Scientific, Mumbai, India) and the picture of the gel was collected. The amplified products were extracted from the gel bands using QIAquick Gel Extraction Kit (QIAGEN). Sequencing of the PCR amplified products was carried out through Sanger sequencing method. The homologous sequences were retrieved using BLAST tool of NCBI and phylogenetic tree was constructed with the help of MEGA 6 and MEGA 7 software [19]**.**

ii. Sequencing PCR, clean up and Sanger sequencing

The sequencing PCR recipe for each 5 µl volume reaction was: Buffer- 1.0 µl; Big Dye- 1.0 µl; primer- 0.5 µl; and template DNA- 2.5 µl. The cycling conditions used for sequencing PCR were: 96°C- 1 min; 96°C - 10 sec; 52°C- 40 sec; 60°C- 4min; 4°C- ∞. The cycles were repeated till 35 times. After the PCR, the samples were cleaned up and prepare for Sanger sequencing. The PCR reaction products were transferred into sterile 1.5 ml microfuge tube. Master Mix I consists of 10 µl Mili-Q and 2 µl of 125 mM EDTA per reaction was prepared. Then 12 µl master Mix I was added to each PCR reaction product and contents were mixed properly. After that, Master Mix II consists of 2 µl of 3M NaOAc (pH 4.6) and 50 µl of ethanol per reaction was prepared. 52 µl of master mix II was added to each reaction. The contents were mixed well and incubated at room temperature for 15 mins. Then the reaction mix was spun at a speed of 12000g for 20 mins at room temperature and the supernatant was decanted. Then 250 µl of 70% ethanol was added and span at 12000g for 10 mins at room temperature, the supernatant was decanted. At last12-15 µl of Hi-Di form amide was added, transferred to sample tubes, covered with septa, denatured, spot chilled and proceeded for capillary electrophoresis.The sequences were retrieved using BLAST tool and aligned using CLUSTALW tool in order to find homologous sequences. Phylogenetic tree was constructed using MEGA 6 and MEGA 7 software by neighbour joining method.

2.3.7.2 Cellulase assay

The bacterial isolates were tested for the production of extracellular cellulase. Carboxy Methyl Cellulose (CMC) agar (1%) plates were prepared and allowed to dry properly for about 1 hour. To the center of the plate, the bacterial colonies were spotted with the help of a sterile toothpick. The plates were then incubated at 28^0C for 3-4 days. Bacterial colonies were washed off with sterile distilled water. Then the medium surface were flooded with 0.1% of Congo red solution and incubated at room temperature for 24 hours. Congo red solution was removed, followed by surface washing with sterile distilled water. Surface of the medium was then dipped in 1M sodium chloride (NaCl) solution and kept undisturbed for 2 hours. After removal of NaCl, the plates were investigated for the presence of white clear zones [30].

2.3.7.3 Twitching motility study

As several phytopathogenic bacteria possess twitching motility for penetration and colonization inside its host, it was also studied for the isolated endophytes. For this study,BG plates were prepared and allowed to dry for about 1 hour. Then the saturated bacterial culture of the endophytes were serially diluted up to10^{-6} dilution using following procedure: 100 µl of saturated bacterial culture was added to 900 µl of sterile distilled water in 1.5 ml microfuge tube and mixed properly such that the concentration of the cells becomes 10^{-1}. From this tube, 100 µl of the diluted culture was transferred to another tube with 900 µl of sterile distilled water and mixed properly which makes the concentration 10^{-2}. The process was repeated till the concentration of the cells became 10^{-6}. 2 µl of the diluted culture was spotted on BG agar plate. The plates were allowed to dry, followed by overnight incubation at 28°C. Plates were observed for twitching motility under microscope (EVOS FL, life technologies) at 10X magnification after 10-12 hours of growth [31].

2.3.8 *In vivo* bio-control assay of *Pseudomonas putida* N4T against F1C1 in tomato seedlings

Six to seven days old tomato seedlings germinated under controlled condition were recruited for this study. Freshly grown F1C1colony was inoculated into 20mL BG broth media and incubated at 28ºCand 150 rpm for 36hours. The bacterial cultures were obtained by centrifugation at 4000 rpm and 4 °C for 15 min and were then re-suspended in an equal volume of sterile distilled water to obtain a concentration of approximately 10^9CFU mL−1. Similarly, N4T strain was also inoculated in 20mL BG broth and incubated at 28ºC and 150rpm. After 24 hours N4T culture was centrifuged at 4000 rpm for 15 minutes at 4 °C. The bacterial pellets were re-suspended in sterile distilled water to obtain a concentration of approximately10^9CFU mL−1. The experiment was carried out by root inoculation in a hydroponic condition as described by Singh et al., (2018). For the bio-control assay six treatment sets were established as follows: 1. F (Tomato seedlings were inoculated with F1C1 only on the first day); 2. F+W (Tomato seedlings were inoculated with F1C1 first day and then the next day was re-inoculated with water); 3. W+F(Tomato seedlings were pre-inoculated with only sterile distilled water on first day and on the next day inoculated with F1C1); 4. N+F (Tomato seedlings were pre-inoculated with N4T on first day and then on the next day inoculated with F1C1); 5. F+N

(Tomato seedlings were pre-inoculated with F1C1 and on the next day inoculated with N4T) and 6. W (Tomato seedlings were inoculated with sterile distilled water. After root inoculation each seedling was transferred to the microfuge tubes and kept for ~5 minute, before adding approximately 1mL of sterile distilled water in each tube containing seedlings. Apart from set 1 and 6, all the seedlings after the second inoculation were transferred in to new microfuge tube and kept for 5 minutes before adding approximately 1mL of sterile distilled water. All the inoculated seedlings were transferred to growth chamber maintained at 28°C, 80% RH and 12 hours photoperiod. The seedlings were further investigated for disease progression and from next day onwards data were recorded till 7 days post inoculation. In each set 30 seedlings were employed and each treatment assay was performed in triplicates [21,23, 24-26].

2.3.9 *In vivo* antagonistic activity of theendophytes against F1C1 infection in tomato seedlings

The five endophytes exhibiting*in vitro* F1C1 antagonistic activity were tested for the bio-control efficacy against F1C1 infection in tomato seedlings (cv. Durga Pusa Ruby) grown in a controlled environment [20-23]. The bio-control assay was performed by mix inoculation of the pathogen F1C1 with the endophytes. F1C1 was co-inoculated individually with the endophytes also all the endophytes were inoculated together with F1C1. The experiments were carried under hydroponic condition. The inoculation of the each treatment was done by two methods 1. Root inoculation [21, 23] and 2. Leaf clip inoculation [20, 22]. The bio-control assay was standardized first using only one antagonistic endophyte N4T, further characterized as *Pseudomonas putida* N4T and F1C1.F1C1 was mixed with different volume of N4T and inoculated in tomato seedlings by both leaf inoculation (Kumar et al., 2017) and root inoculation method (Singh et al., 2018). The treatment sets included (1) F1C1+ N4T mix inoculation at volume ratio 1:1, (2) F1C1+ N4T mix inoculation at volume ratio 1: 10, (3) F1C1+ N4T mix inoculation at volume ration 1: 50. Apart from these sets F1C1 was inoculated separately by adjusting the volume with water and N4T was inoculated separately as control. Seedlings inoculated with sterile distilled water were inoculated as negative control. After the experiment performed repeatedly, the F1C1 and endophytes volume ratio were maintained 1:50 for further bio control assays used by all the five endophytes.

The bio-control assay through root inoculation included different treatment sets as follows (1) F1C1+ N4T (50x), (2) F1C1+ C6a (50x), (3) F1C1+ C10 (50x), (4) F1C1+ C11 (50x), (5) F1C1+ C12 (50x), (6) F1C1+ *E. coli* (50x) (7) F1C1 + all (10x) and (8) F1C1, the volume of F1C1 was adjusted by adding water. The bacterial culture was prepared by inoculating F1C1 and the isolates separately in 40 ml BG broth medium followed by incubation at 28 °C. The concentration of bacterial cell was adjusted to $\sim 10^9$ CFU/ml. Each broth culture was centrifuged at 4000rpm for 15 min, then the supernatant were discarded and the cells were collected. Cells were washed twice and were re-suspended using sterile distilled water. Then the treatment sets were prepared by mixing the isolates with F1C1. 200 µl of F1C1 were mixed with 10ml of endophytes in each case. In one set, 2ml of each endophytes were mixed with 200µl F1C1. Six to seven days old seedlings were employed for the study and inoculated with prepared mixed inoculums through root inoculation method. The seedlings were kept in growth chamber (Scigenics, India), andmaintained at 28 ºC temperatures, 80% Relative Humidity and a photoperiod of 12 h. Each treatment consisted of 30 seedlings. The experiment was performed in triplicates and up to 8 days post inoculation each day the data for disease progression were recorded.

Similarly, for leaf clip inoculation the experimental sets were prepared by using fresh grown cultures of F1C1 and all the isolates. The treatment sets were prepared as mentioned above. All the isolates were mixed with F1C1 individually and also in a set together as mentioned above. The seedlings were inoculated by leaf clip inoculation with the help of sterile scissor. After the inoculation the seedlings were analyzed for disease progression and data were recorded for further statistical study. For leaf clip inoculation the data were recorded up to 10 days post inoculation. The experimental sets were performed in triplicates.

2.4 Results

2.4.1 Bacteria isolated from tomato seedlings exhibits*R.solanacearum*F1C1 antagonistic activity in vitro

A total of twenty one bacteria were isolated from tomato seedlings grown in controlled condition. All the isolates were tested for their antagonistic activity against F1C1. Five isolates viz. N4T, C6a, C10, C11 and C12 were found to inhibit F1C1 both by agar well diffusion and cross streak method. These five isolates were investigated for their

antagonistic activity against each other. One isolate C10 did not exhibit any antagonistic activity against other four isolates. On the contrary, the other fourisolates showed antagonistic activity at least against one isolate. The highest antagonistic activity was of N4T against C10. The diameter of inhibition zone was measured as 30mm. C6a was found to inhibit N4T with very minute inhibition zone. C11 showed very minute inhibition zone against C6a and C12. C12 was found tom inhibit N4T with a very minute inhibition.

Table 2.2 : Measurement of Inhibition zone of the isolates against F1C1 studied by agar well diffusion method. Five bacteria namely N4T, C6a, C10, C11, C12 found to inhibit F1C1 on agar plate. The mean value was calculated, ± symbol indicates values for standard deviation of means.

Isolate	Inhibition zone (mm)
N4T	16.16 ± .28
C6a	16 ± 1
C10	15.33 ± .57
C11	15.66 ± .57
C12	15.66 ± .57

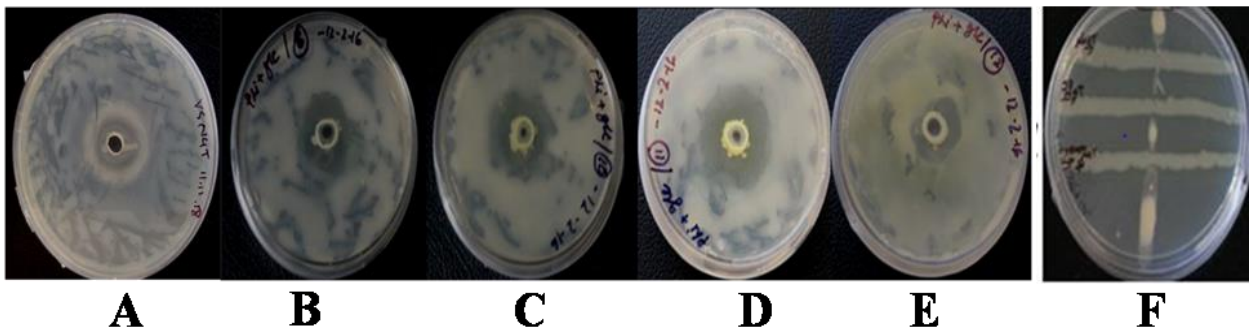

Fig 2.3: In vitro antagonistic test by agar well diffusion and Cross-streak method: A-E) Bacterial isolates N4T, C6a, C10, C11 and C12 respectively exhibiting inhibition zone against F1C1 in agar well diffusion assay respectively. F) N4T showing antagonistic activity against F1C1 by Cross- streak method.

Table 2.3 : Interaction of F1C1 antagonistic isolates among each other

Wells ↓	Spread →					
	F1C1	N4T	C6a	C10	C11	C12
N4T	++	-	-	++	-	-
C6a	++	+	-	-	-	-
C10	++	-	-	-	-	-
C11	++	-	+	-	-	+
C12	++	+	-	-	-	-

++ indicates large inhibition zone (> 10mm), + indicates minute inhibition zone

Wells indicates bacteria placed in wells, spread indicate bacteria spread in the plate.

Arrow marks indicates the direction

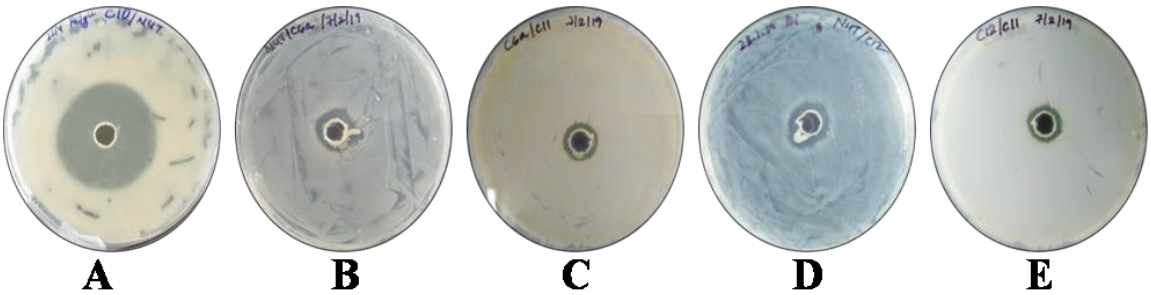

Fig 2.4: Interaction study between F1C1 antagonistic isolates by agar well diffusion method. A) A large clear zone indicates N4T is inhibiting C10; B) C6a inhibits N4T; C) C11 inhibits C6a; D) C12 inhibits N4T, E) C11 inhibits C12

2.4.2 Identification of all the bacterial isolates

All the 21`bacterial isolates including the five F1C1 antagonistic bacteria were subjected to 16S rDNA sequencing in order to molecular characterization. The five F1C1 antagonistic bacteria were identified N4T as *Pseudomonas putida*, C6a as *Bacillus subtillis*, C10 as *Enterobacter* spp., C11 as *Burkholderia* spp. and C12 as *Burkholderia* spp. Out of the twenty one isolates maximum six were belong to *Klebsiella* genus, five were from *Burkholderia* genus, four from *enterobacter*, two were from *Bacillus* and one

each were found to be from *Pseudomonas*, *Delftia*, *Serratia* and *Sphingomonas* genus (Table).

2.4.3 Cellulase activity and twitching motility

All the bacterial isolates were tested for their extracellular cellulase activity and for twitching motility. Out of the 21 isolates, only 3 were found to be exhibiting very low cellulase activity on CMC agar plate. Rests of the isolates were not showing cellulase activity. All the three isolate viz N1, N5 and W4 were belong to *Klebsiella* species. Whereas none of the isolates were found to be twitching motility positive (Table)

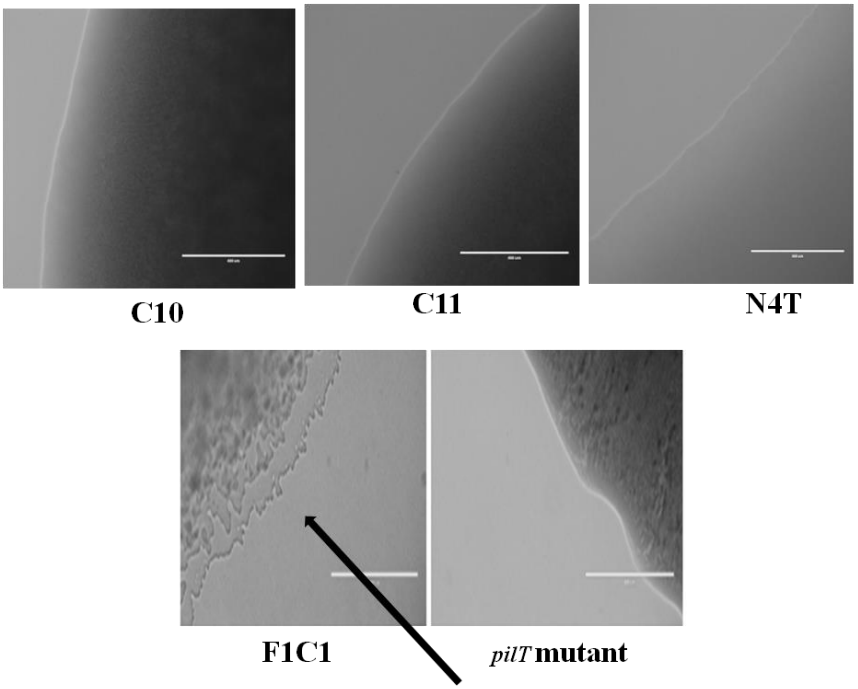

Fig 2.5: Twitching motility of bacterial isolates: From left isolates C10. C11 and N4T are not exhibiting twitching motility. All of these three isolates inhibit *R. soalnacearum* F1C1. F1C1 which is known to exhibit twitching motility is compared with all the isolates. While F1C1 was showing twitching motility the *pilT*mutant of F1C1 which was deficient in twitching motility was taken as negative control. All the twenty one isolates were studied for twicthcing motility and none of them were proficient for twitching motility

Table 2.4 : Characterization of tomato seedling bacterial isolates

Sl.No.	Bacterial isolates	Cellulase activity	Twitching Motility	Pathogenicity in tomato seedlings	16S rDNA Identification
1.	N4T	Absent	Absent	Negative	*Pseudomonas putida*
2.	C6a	Absent	Absent	Negative	*Bacillussubtilis*
3.	C10	Absent	-do-	-do-	*Enterobacter* spp.
4.	C11	Absent	-do-	-do-	*Burkholderia* spp.
5.	C12	Absent	-do-	-do-	*Burkholderia*spp.
6.	C6b	Absent	-do-	-do-	*Burkholderia* spp.
7.	Endo 1	Absent	-do-	-do-	*Sphingomonas spp.*
8.	Endo 2	Absent	-do-	-do-	*Bacillusmegaterium*
9.	Endo 5	Absent	-do-	-do-	*Burkholderia* spp.
10.	Endo 9	Absent	-do-	-do-	*Enterobacter cloacae*
11.	N1	Very low	-do-	-do-	*Klebsiella* spp.
12.	N3W	Absent	-do-	-do-	*Enterobacter* spp.
13.	N4	Absent	-do-	-do-	*Klebsiella*spp
14.	N5	Very low	-do-	-do-	*Klebsiella* spp.
15.	N6	Absent	-do-	-do-	*Burkholderia*spp
16.	W1	Absent	-do-	-do-	*Klebsiella variicola*
17.	W2	Absent	-do-	-do-	*Klebsiella*spp
18.	W3	Absent	-do-	-do-	*Enterobacter* spp.
19.	W4	Very low	-do-	-do-	*Klebsiella spp.*
20.	W7B	Absent	-do-	-do-	*Serratia* spp.
21.	W7S	Absent	-do-	-do-	*Delftia*spp

2.4.4 Bio-control assay using *P. putida* N4T and effect of water in F1C1 root inoculation

The efficacy of *P. putida* N4T for the control of the disease caused by F1C1 in tomato seedlings was evaluated using root inoculation method described by Singh et al. (2018). In this study seedlings were pre-inoculated with N4T and then F1C1 was inoculated after 24 hours also the vice versa was done to find out the disease progression and the bio-control efficacy of N4T against F1C1 infection. The treatments were compared with seedlings only inoculated with F1C1.The occurrence of wilting symptoms was delayed and significant reduction of seedling death was observed in the treatment group “N+F”

seedlings pre-inoculated with N4T. The percentage of seedlings death in the group was 27.77% after seven days post inoculation, which was very low in compare to the control groups where F1C1 was pre-inoculated. In the control group “F” where seedlings were only inoculated with F1C1, 84.44% seedlings were wilted. In another control group “F+W” seedlings pre-inoculated with F1C1 and then next day exposed to water, 78.88% seedlings were wilted. Interestingly the wilting symptoms or the percentage of death seedlings were not reduced in treatment “F+N” seedlings pre- inoculated with F1C1 and then inoculated with N4T on the next day. In this group 81.11% seedlings were wilted.This signified that N4T could control the disease caused by F1C1 effectively when pre-inoculated in the seedlings, but not after inoculation of F1C1 in the seedlings.

In another treatment “W+F” in which root of the seedlings were exposed to water and then inoculated with F1C1 significant reduction of the disease was observed. In this treatment only 42.22% seedlings were wilted. This implies that water had an impact on the disease caused by F1C1 via root inoculation. Similar observation was reported previously in another study by Singh et al. (2018). The water impact on the disease progression

Table 2.5 : Details of treatment sets for bio-control efficacy of N4T against F1C1 in tomato seedlings using pre inoculation root dip method.

Treatment	**Treatment details**	**% of killed seedlings**
F	Only F1C1 inoculated	84.44
F+W	First F1C1 then water was inoculated	78.88
W+F	Seedlings were pre-exposed with water then F1C1 added	42.22
N+F	Seedlings pre-inoculated with N4T , then inoculated with F1C1	27.77
F+N	Seedlings pre-inoculated with F1C1 then inoculated with N4T	81.11
W	Water control	0

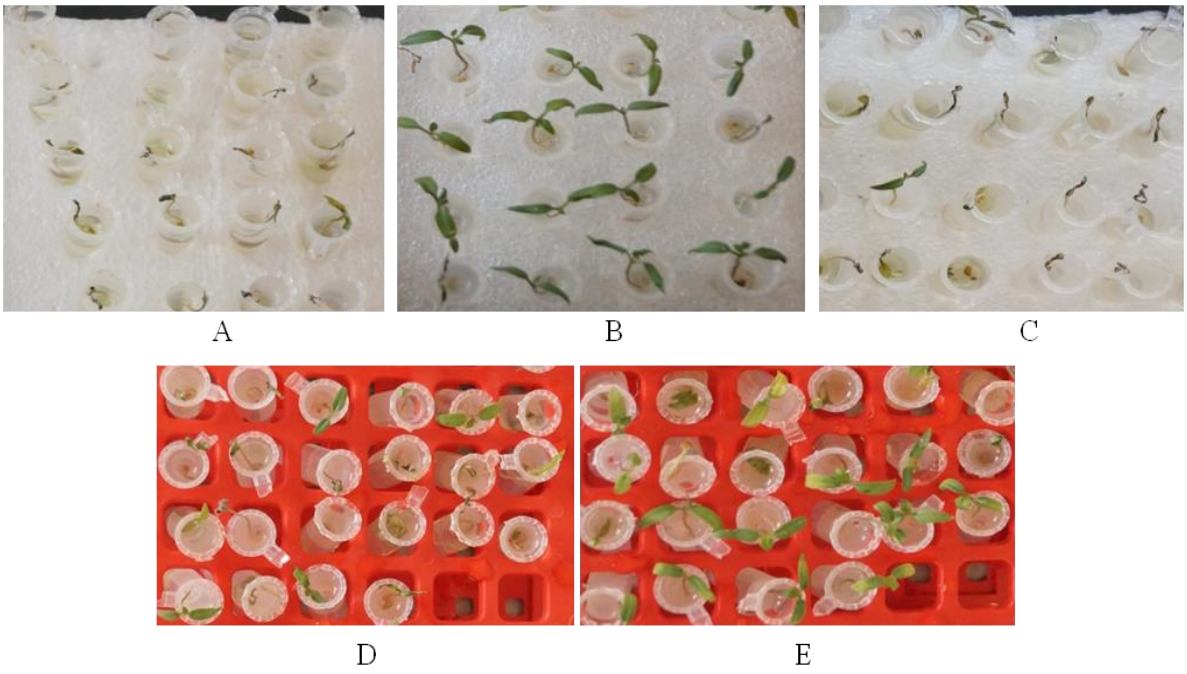

Fig 2.6: Control efficacy of N4T against F1C1. A) Tomato seedlings pre-inoculated with F1C1 and then inoculated with N4T; B) Tomato seedlings pre-inoculated with N4T and then inoculated with F1C1; C) Tomato seedlings pre-inoculated with F1C1 and again exposed with water next day; D) Tomato seedlings inoculated with F1C1; E) Tomato seedlings pre-inoculated with water and then inoculated with F1C1. Pictures were taken after 7days post inoculation.

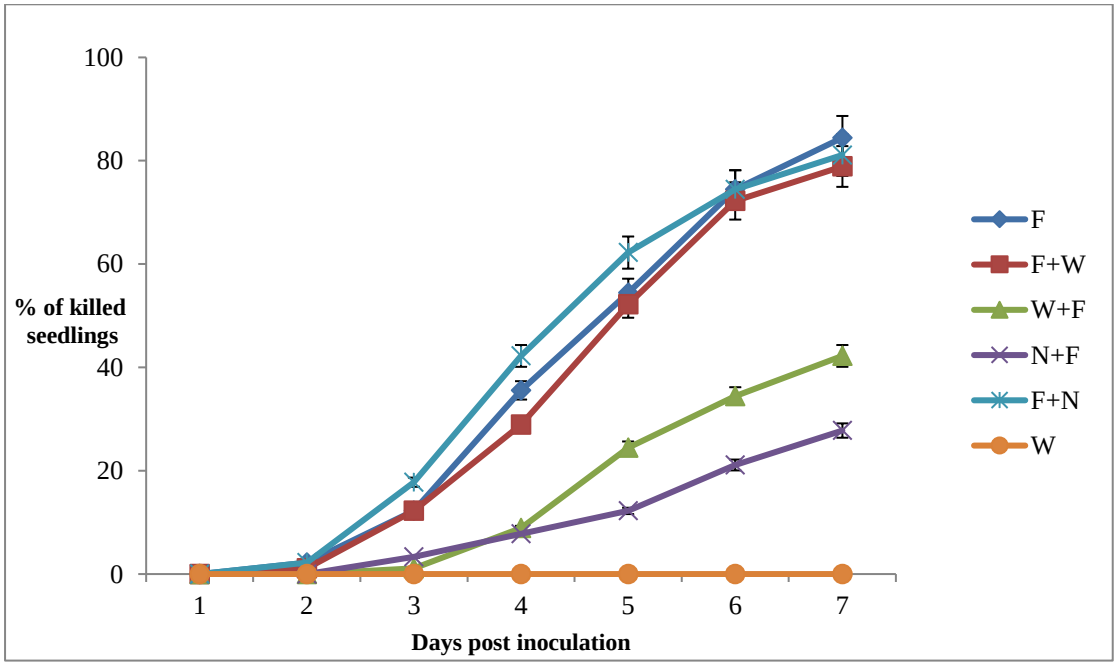

Fig 2.7: Bio-control efficacy of N4T against F1C1 infection. X-axis represents days post inoculation, and Y- axis represents the percentage of wilted seedlings.

2.4.5 In planta bio-control assay of the five endophytes using mix-inoculation

A mix-inoculation method was standardized for the bio-control assay and to attain maximum protection, the endophytes were mixed in higher volume with low volume of the pathogen F1C1. The endophytes were individually mixed with F1C1 also they were mixed together with F1C1 to find out the effective bio-control against the disease. The inoculation in the tomato seedlings were done by root inoculation [21, 23] and leaf inoculation [20, 22] method. Both in the root inoculation and leaf inoculation all the five endophytes was found to effectively control the disease individually as the number of wilted seedlings were significantly reduced in the treatments with the endophytes. Also in the treatment set where all the five endophytes were inoculated together against F1C1, the numbers of wilted seedlings were effectively controlled in both the method. In the root inoculation 68.89 % seedlings were killed in F1C1 control set, whereas the lowest number of seedlings killed were foundin the treatment F+ all, in which all the endophytes were inoculated together with F1C1 with only 2.22% killed seedlings.In the treatment F+N4T, where N4T was inoculated with F1C1 only 6.67% seedlings were killed. In the treatment F+C6a, where C6a was co-inoculated with F1C1, only 4.44% seedlings were killed. In the treatment F+ C10, in which C10 was co-inoculated with F1C1, 17.78% seedlings were killed. In the treatment F+ C11, in which C11 was co-inoculated with F1C1, only 4.44% seedlings were killed. In the treatment F+ C12, in which C12 was inoculated with F1C1 only 6.67% seedlings were killed. To compare the mix inoculation of the endophytes with F1C1 in one set mix culture of F1C1 and E. coli MG1655 strain was treated in the seedlings where it was found that more than 40% seedlings were killed.

In the leaf clip inoculation the maximum protection was found in the set where F1C1 was mixed with C11 and was inoculated by leaf clip method, only 4.44% of the seedlings were died. In the set in which only F1C1 was inoculated 75.56% seedlings were died. It was found that apart from C11 the other four isolates N4T, C10, C6a and C12 were also effective in reducing the number of the diseased seedlings. Also when all the isolates were mixed together with F1C1 and inoculated through leaf clip method the disease was reduced significantly, only 8.89% of the seedlings were died in this treatment set.

Table 2.6 : Root dip and leaf clip mix inoculation to study biocontrol efficacy of the five bacterial endophytes

Treatment	**Root inoculation (% of killed seedlings)**	**Leaf clip inoculation (% of killed seedlings)**
F1C1	68.89	75.56
F1C1+ N4T (50x)	6.67	14.44
F1C1 + C6a (50x)	4.44	11.11
F1C1+ C10 (50x)	17.78	6.67
F1C1+ C11 (50x)	4.44	4.44
F1C1+ C12 (50x)	6.67	18.89
F1C1+ all (10x)	2.22	8.89

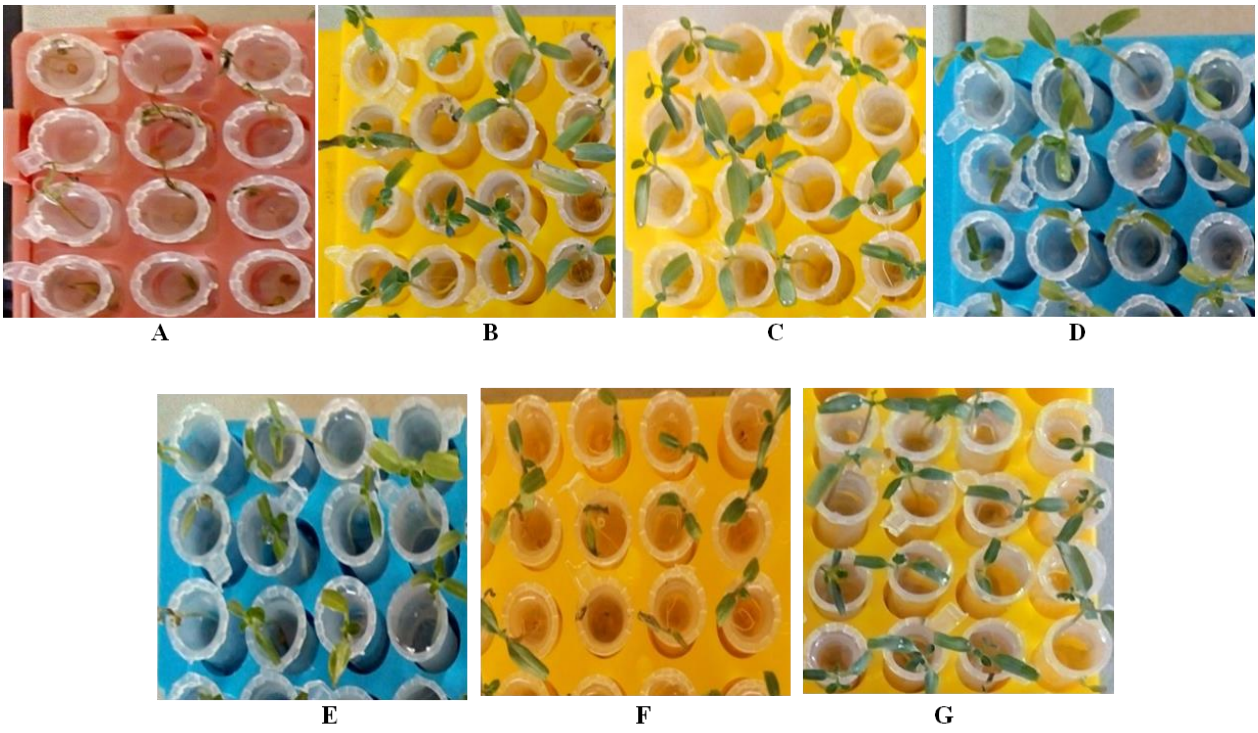

Fig 2.8: Effect of different treatments against F1C1 after 10 days of mixed leaf clip inoculation. A) F1C1 inoculated seedlings, B) F1C1+N4T (50x), C) F1C1+ C6a (50x), D) F1C1+ C10 (50x), E) F1C1+ C11 (50x), F) F1C1+ C12 (50x), G) F1C1+ all the five anti-F1C1 isolates (10x each)

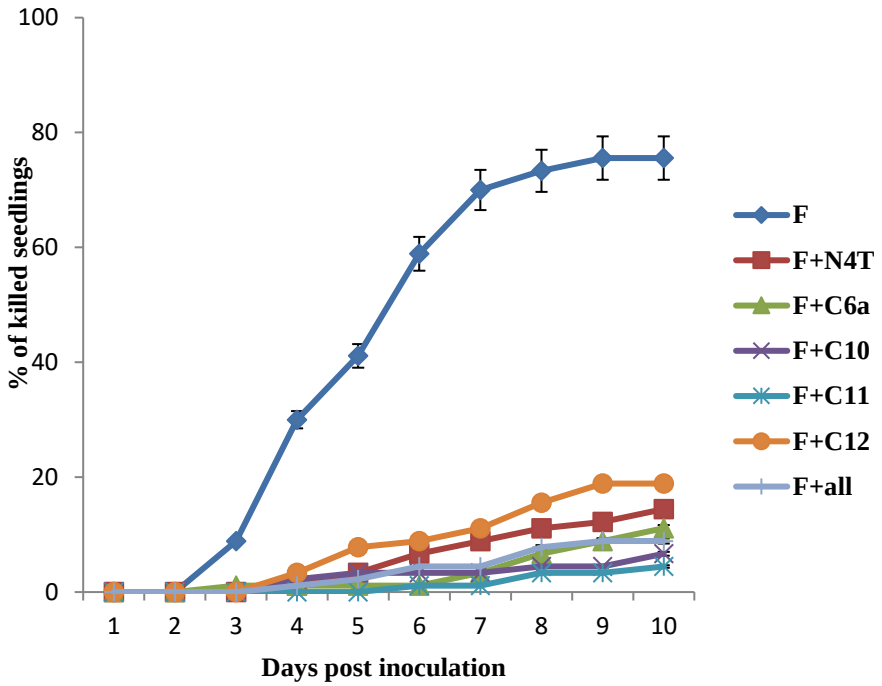

Fig 2.9: Bio-control efficacy of the five bacterial isolates individually and by consortia through mix inoculation by leaf clip method against F1C1 infection. X-axis represents days post inoculation, and Y- axis represents the percentage of wilted seedlings

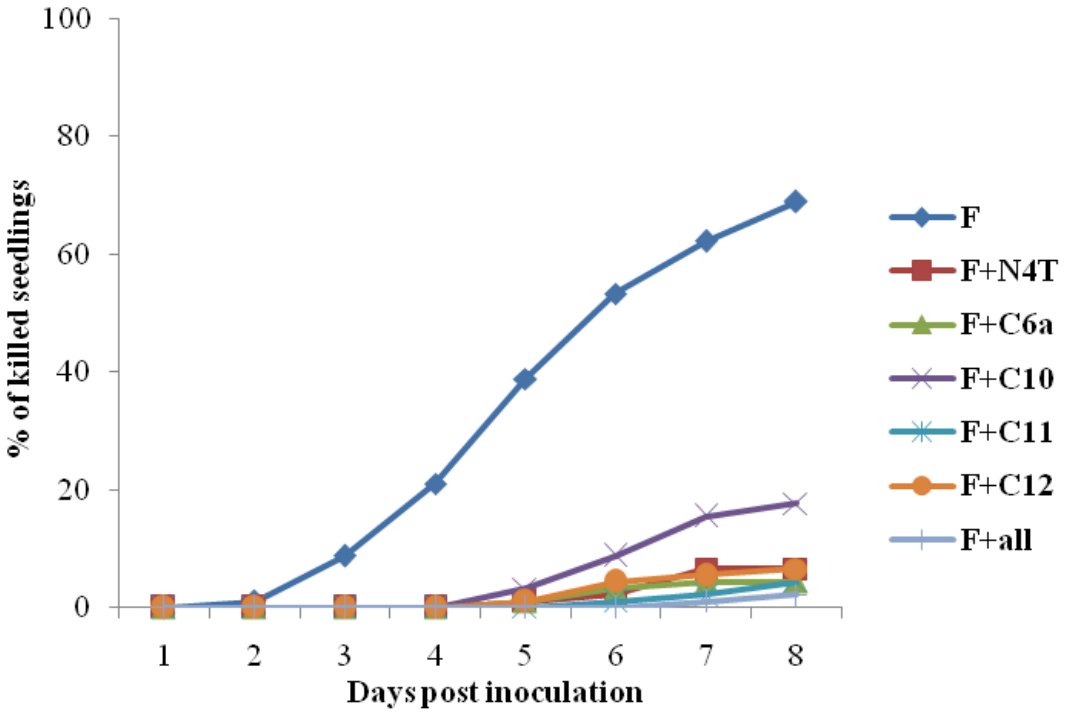

Fig 2.10: Bio-control efficacy of the five bacterial isolates individually and by consortia through mix inoculation by root dip method against F1C1 infection. X-axis represents days post inoculation, and Y- axis represents the percentage of wilted seedlings

2.5 Discussion

The interaction of plant pathogen with its host and other resident endophytes has been a important area of research in recent years. Some of the pathogens are so aggressive that invades and hijackes the host plant and ultimately kills the plant. In recent years many such plant pathogens has been studied rigorously and due to the advancement of technologies, the understanding of this pathogens are better than ever. But only understanding plant pathogen is not enough to fully comprehend the behavior of the pathogen. There are different level of interaction between host plant and pathogen as well with other host associated microbes and environmental factors exists, which yet to befully explored. The pathogenic natures of microbes are attained through many mechanisms and many molecular weapons are involved throw out the infection process. Pathogen deploys different molecular determinants in different stages of infection. During the time of infection the pathogen has to encounter different defenses of plant but there are interactions between the native endophytes with the pathogen and host. Several plant beneficial endophytes has been studied for efficacy in control the disease caused by plant pathogens. Although several endophytes have been found to be control disease caused by plant pathogens, deep investigation on complex dynamics of the plant-pathogen-endophyte-environment interaction is much required.

Ralstonia solanacearum is a soil borne bacterial plant pathogen that causes lethal wilt disease in many plants including economically important plants like tomato, brinjal, potato, chili etc [13, 14]. To mitigate the disease caused by the bacterium is a herculean task as many of the control methods that have been used are not adequate. Using of biological control agents against *Ralstonia solanacearum* can be an alternative approach to manage the disease due to the environmental friendly nature.

In recent studies, the author's laboratory has developed different methods to study the pathogenicity of *R. solanacearum* in seedling stages of tomato and brinjal [20-23]. These methods were designed such a way that during the time of *R. solanacearum* infection the presence of outside microorganism is minimal. The methods developed in the laboratory were found to be effective in studying *R. solanacearum*pathogenicty. It was observed that there was no symptom appeared in few of the seedlings inoculated by the pathogen and the seedlings somehow escaped the disease. It was intriguing to unravel if the resident endophytes of the seedlings have some role against the pathogen and if there are

any *R. solanacearum* antagonistic bacterial endophytes can be isolated from the tomato seedlings. With this outlook, in this study , different culturable endophytic bacteria were isolated from six to seven days old tomato seedlings grown in controlled environment. Based on the colony morphology total 21 colonies were obtained for further study. First, all the isolates were tested against *R. solanacearum* F1C1 strain for antagonistic activity in vitro by agar well diffusion and cross streak method. Out of the 21 isolates, 5 were found to be inhibiting *R. solanacearum* F1C1 growth in vitro.All the 21 isolates were molecular characterized by 16S rDNA sequencing. The bacteria were belonging to 8 different genus including *Klebsiella, Burkholderia ,Enterobacter, Bacillus,Pseudomonas, Delftia*, *Serratia* and *Sphingomonas*. Further all the isolates were tested for their pathogenicity in tomato seedlings by re-inoculating them in the seedlings. None of the isolates showed any symptoms to the seedlings.

To find out if the 5 F1C1 antagonistic isolates can inhibit each other they were tested against each other using in vitro approach. Isolate C10 which belongs to *Enterobacter*spp did not show any inhibition to the other 4 isolates and found to be specific against the pathogen F1C1. Isolate N4T belonging to *Pseudomonas putida* species could inhibit C10 apart from inhibiting F1C1. C6a belonging to *Bacillus subtilis* showed very minute inhibition againstN4T apart from inhibiting F1C1. Isolate C11 belonging to *Burkholderia* spp. was found to exhibit minute inhibition against C6a and another isolate C12 which is belongs to *Burkholderia* spp. Then C12 was found to be able to inhibit N4T. This study suggested that the bacterial endophytes apart from inhibiting pathogenic F1C1 strain also can inhibit each other. It is assumed that these isolates might interact among each otherin plant also and some of the isolates might inhibit other bacteria nearby its vicinity.

It is known that many plant pathogenic bacteria including *R. solanacearum* can degrade plant cellulose by exhibiting extracellular cellulase activity in order to penetrate the internal tissues of the host. But in case of endophytes it is thought be not mandatory in order to colonize internal tissues of the plant. How the endophytes colonize inside plant without degrading plant cell component is not known. The cellulose assay was carried out for all the bacterial isolates and only 3 isolates all belonging to *Klebsiella* spp. were showing minute cellulase activity.

Twitching motility is associated with plant pathogenic bacteria and has been characterized as a pathogenic trait which is driven by type IV pili. Twitching motility is

generally absent in nonpathogenic bacteria. The bacterial wilt pathogen R. *solanacearum* is known to possess twitching motility. In this study, upon tested none of bacteria isolated from tomato seedlings were found to be exhibiting twitching motility.

One of the five F1C1 antagonistic bacteria viz. *P. putida* N4T was tested for bio-control efficacy of the disease caused by F1C1 strain tomato seedlings ina time point experiment. The in planta test confirmed that N4T strain was effective in controlling the disease when it was pre-inoculated in the seedlings. When N4T was pre-inoculated and after one day the pathogenic F1C1 strain was inoculated only 27.77% seedlings were killed where as in the seedlings treated with F1C1 and seedlings first treated with F1C1 and then again exposed with water 84.44% and 78.88% seedlings were killed. This means pre-inoculation of the seedlings with N4T reduced the disease significantly; approximately 50% disease was reduced. In the same study it was found that in the treatment set where F1C1 was first inoculated and then after one day N4T was inoculated there was no protection of the seedlings and N4T was not effective in controlling the disease. The might be due to the rapid progression of the F1C1 inside the seedlings. So once it get inside it is very difficult to control the disease. In another set it was found that when the root of the seedlings was pretreated with water and then F1C1 was inoculated the disease was reduced. The impact of water in F1C1 infection by root inoculation is interesting as this was not mentioned previously by the groups that have carried similar bio-control assay [24, 35]. The study established that the reductions of the disease in the seedlings are mainly by pre-inoculation of N4T in the seedlings also water plays a role in the disease control.

In another study, all the five F1C1 antagonistic isolates including N4T were tested for their efficacy in controlling the disease caused by F1C1 by mix inoculating the pathogen and the isolates individually and altogether through leaf clip inoculation and root dip inoculation. Here, all the isolates were mixed with the F1C1 culture individually at different increasing volume of the isolates, It was observed that when the isolates were mixed in 1:1 ratio with F1C1 and inoculated in the seedlings the disease was reduced, but to get maximum control, the volume of the bacterial isolates were increased. Both by leaf clip mode of mix inoculation and root dip mode of mix inoculation was found to be effective in reducing the disease in tomato seedlings.

The results and observations obtained in this study suggest that bacteria isolated from a host can be used as a potential bio-control agent in the same host against pathogen like *R. solanacearum*. Further detailed study will illuminate more on the mechanisms of antagonism of these isolates and more study is required to use these bacteria as bio-control agent in the field.

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