CHAPTER 3

CHARACTERIZATION OF *PSEUDOMONAS PUTIDA* N4T AND STUDY OF ITS EFFECT IN CONTROL OF BACTERIAL WILT IN GROWN-UP TOMATO PLANTS AND IN BRINJAL SEEDLINGS

3.1 Abstract

Pseudomonas putida N4T is a bacterial endophyte isolated from healthy tomato seedlings grown under gnotobiotic conditions. It exhibits antagonistic activity against a bacterial phytopathogen *Ralstonia solanacearum* F1C1 which causes lethal wilt disease in different plants, and against another bacterial endophyte *Enterobacter* C10. In this study, the N4T strain was characterized, and also its ability to colonize inside tomato seedlings, brinjal seedlings, and grown-up tomato plants with the help of GFP tagged strain of N4T was investigated. It was established that N4T can colonize vascular tissues of tomato seedlings and grown-up tomato plants and it can also colonize inside the brinjal seedlings. Further, the efficacy of N4T in controlling the disease caused by F1C1 in grown-up tomato plants and brinjal seedlings were examined. The N4T strain was found to be effective in controlling the disease caused by F1C1 in grown up tomato seedlings and brinjal seedlings. Also, the role of a gene *gacA*, a response regulator which is a part of two component regulatory system present in N4T was studied against F1C1 and C10. The role of *gacA* in various biological functions including antibacterial activity has been elucidated in several *Pseudomonas* strains. In this work, we created an insertion mutation in *gacA* gene homolog of N4T strain using Calcium chloride treatment and followed by heat shock transformation. Upon tested against F1C1 and C10, it was found that the antagonistic activity of *gacA* mutant was intact against F1C1 but the antagonistic activity was deficient against C10. This suggests that the *gacA* gene plays role in the inhibition of C10, but does not have any role in the inhibition of F1C1.

3.2 Introduction

Pseudomonas spp. is a Gram-negative, highly versatile, and ubiquitous bacteria that are of great interest because of its various potential biotechnological applications and also for its ability to cause disease in humans and plants. Among others, *Pseudomonas putida* are well-studied bacterium that has been elucidated for various roles and can be found

throughout various environments [1-9]. *P. putida* is known to possess a wide range of metabolic activities including plant growth promotion, degradation of organic solvents from pollutant environments, production of different bioactive metabolites, etc. [10-12]. *P. putida* has also been studied for its role in suppressing plant pathogens and protection of plants from various diseases [13-17]. Researchers have established the presence of *P. putida* in the rhizosphere and endosphere of various plants [18-20]. Although the importance of *P. putida* in various biotechnological applications has been studied the detailed mechanisms of these functions and interaction with other microbes, and environments are poorly understood.

In the previous chapter, different bacterial endophytes were isolated from healthy tomato seedlings and were tested against the phytopathogen *Ralstonia solanacearum* F1C1 strain, a causal agent of bacterial wilt [21]. Among the twenty-one bacterial isolates, five were found to be inhibiting F1C1 in vitro and all five of them were tested for their efficacy in reducing the disease caused by F1C1 in tomato seedlings. In this chapter one F1C1 antagonistic bacterium, *Pseudomonas putida* N4T was recruited and was characterized for different traits. The N4T strain was studied for its stable colonization in tomato and brinjal seedlings and also its ability to reduce the disease symptoms caused by F1C1 was elucidated in both hosts. In the previous chapter, it has been found that apart from inhibiting F1C1, the N4T strain could inhibit another F1C1 antagonistic strain *Enterobacter* C10. In this study, a gene *gacA* from N4T was studied for its role in antibacterial activity against F1C1 and C10. The *gacA* gene is a response regulator and a part of a two-component regulatory system GacS/GacA. The GacS/GacA system plays a crucial role in the regulation of a wide range of biological functions in many bacteria including different *Pseudomonas* strains. For example, this system regulates genes responsible for virulence traits in a plant pathogenic strain *P. syringae* pv. tomato DC3000 [22]. In the case of the human opportunistic pathogen *P. aeruginosa*, the GacS/GacA system was found to be negatively regulating T3SS expression and motility, and positively regulating bio-film formation and type VI secretion [23]. In some other *Pseudomonas* strains, the system has been found to regulate genes that impart antibacterial activity. Zhang et al. established that *gacA* gene of *Pseudomonas fluorescens* FPT9601 plays important role in the suppression of bacterial wilt disease caused by *R. solanacearum* [24]. On the contrary, Alit-Susanta et al. found that GacS/GacA system of bio-control strain *P. fluorescens* PfG32R does not have any role in

antibacterial activity against *R. solanacearum* and *Clavibacter michiganensis ssp. Michiganensis* [25]. In this work, an insertion mutation in *gacA* gene of N4T strain was created by using a modification of the $CaCl_2$ treatment and heat shock method. The *gacA* mutant was studied against F1C1 and C10 in vitro using an agar well diffusion assay for antagonistic activity and was compared with wild-type N4T.

3.3 Materials and Methods

3.3.1 Bacterial strains, growth media, and culture conditions

Bacterial strains used in the entire work have been listed in Table 1. The growth medium used for the wild-type *R. solanacearum* F1C1 [21] and derivative mutant strains, as well as *Pseudomonas putida* N4T, was BG (Bacto agar-glucose) medium [26] supplemented with 0.5% glucose at 28°C. Agar (15 g/L) was added for plating as and when required. *Escherichia coli*DH5αstrain was grown on Luria-Bertani (20 g/L) medium at 37 °C and whenever required Agar (15 g/L) was added for plating purposes. Antibiotics used in the experiments were ampicillin, gentamycin, and spectinomycin which were added to the medium at a concentration of 50 μg/ml, as per required. The media components and the antibiotics used in this study were procured from Hi-media.

E. coli DH5α competent cells were prepared following Inoue *et al.*

Table 3.1: Bacterial strains used in this study

Sl No.	**Strain**	**Characteristics**	**Reference/Source**
1	F1C1	Wild type virulent *R. solanacearum* strain (phylotype I), was isolated from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India	[21]
2	N4T	*Pseudomonas putida*, bacterial isolate from tomato seedlings, exhibit antagonistic activity against F1C1 and C10	Lab collection
3	DH5α	F− recA lacZ ΔM15	Lab collection

4	TPP10	Amp^r; and Gent^r;*gacA*mutant, deficient in inhibiting C10, but proficient for inhibition of F1C1, derived from N4T	This work
5	C10	*Enterobacter* species, bacterial isolate from tomato seedlings, exhibit antagonistic activity against F1C1, but N4T inhibits it	Lab collection
6	TPP11	Kan^r;the strain of N4T having pDSK-GFPuv plasmid vector,	This work
Plasmid / construct vector			
1	pTZ57R/T	Amp^r; Cloning vector	Thermo Scientific
2	pCZ367	Amp^r; Gent^r; Insertional vector with lacZ reporter	[27]
3	pPLST1	pTZ57R/T::$gacA_{N4T}$	This work
4	pPLST001	pCZ367:: $gacA_{N4T}$	This work
5	pDSK-GFPuv	Kan^r; constitutively expressed*gfp*	[28]
This table contains the details of bacterial strains, plasmid vectors, and vector constructs that are used in this work.			

3.3.2 Germination of Tomato and Brinjal seedlings

Germination of tomato seedlings was done by following Singh *et al* [27]. The germination of tomato seedlings was described in Chapter 2. Tomato seeds of Durga Pusa Ruby were presoaked in sterile distilled water for 2 days. This was followed by spreading the seed on sterile wet tissue paper in a plastic tray and allowing them to germinate in a growth chamber (Orbitek) maintained at 28C, 80% relative humidity (RH), and a 12-h photoperiod. Sterile distilled water was sprinkled on a regular basis to sustain the germination process for 6 to 7 days.

Brinjal seeds of Dev Kiran variety selected for this study were pre-soaked in sterile distilled water for 5 to 6 days, and then kept on a sterile wet tissue paper bed in a plastic

tray. After that, the plastic tray containing the seeds were allowed for germination in a growth chamber (Orbitek, Scigenics, India) maintained at 28°C, 80% relative humidity (RH), and 12 h photoperiod respectively. Sterile distilled water was sprinkled at regular time intervals to sustain the germination process till the seedlings are emerges and become ready for the experiments [27, 29]

3.3.3 Competent cells preparation of *P. putida* N4T

P. Putida N4T competent cells were prepared following Chakrabarty *et al.* and Zhao *et al.* with little modifications. *P. putida* N4T was streaked on a BG agar plate. Freshly grown *P. putida* N4T pure colony was added to 20 ml of BG broth with a sterile loop and allowed to grow in a shaking incubator (Orbitek) maintained at 28°C and 150 rpm. A portion of the starter culture was re-inoculated in 50 ml BG broth and allowed to grow at 28°C and 150 rpm shaking conditions. The optical density (OD) was measured time to time and when the OD reached around 0.4 the cells were chilled. Then the cells were harvested by centrifugation and washed with 10mM NaCl. The cells were again centrifuged and re-suspended with 100 mM ice-cold $CaCl_2$. The cells were kept on ice for 30 minutes, harvested by centrifugation, and re-suspended again with 100mM ice-cold $CaCl_2$. The resulting cells were then harvested in a 1.5ml microfuge tube in aliquots and preserved in deep freeze for further use [30, 31].

3.3.4 Creation of GFP tagged *P. putida* N4T and its colonization study in tomato seedlings and grown-up tomato plants

The plasmid, pDSK-GFPuv, containing one copy of constitutively expressed *gfp*, and kanamycin resistance genes was kindly donated by the Plant Biology division, The Samuel Roberts Noble Foundation, USA [28]. This plasmid was introduced to N4T by the Calcium chloride ($CaCl_2$) heat shock transformation method as described by Zhao *et al.*, 2013. N4T cells were treated with 100 mmol L^{-1} of $CaCl_2$ for generating competent N4T cells. The competent N4T cells were mixed with the pDSK-GFPuv plasmid and the plasmid-cell mixture was subjected to heat shock for 3 minutes at 42^{o} C, chilled in ice for 2 minutes and 30 seconds. After that 900 µl of BG broth was added to the mixture and the cells were allowed to incubate for 1.5 hours at 28°C in a shaking incubator. After a short spin, the supernatant was discarded and around 100µl of the cell pellets were spread on a BG plate containing antibiotic kanamycin. Successful transformants were

observed for the emission of green fluorescence under a fluorescence microscope (EVOS, Life Technologies). One successful transformant TPP11 was selected for further studies.

To study the colonization of tagged N4T, six to seven days old tomato seedlings grown under a controlled environment were recruited. The seedlings were inoculated via the root inoculation method as described by Singh *et al*., 2018. Seedlings inoculated with the wild-type N4T were taken as control. The seedlings were kept in a growth chamber at 28^{o} C temperatures, 80% relative humidity, and a photoperiod of 12 h. From the next day onwards of inoculation, the colonization of N4T inside the seedlings was studied. Every day few seedlings were randomly selected and subjected to microscopic visualization. The seedlings were surface disinfected as described by Kumar *et al*., 2013, and the cross-sections of the seedlings or the direct seedlings were observed under a fluorescence microscope (EVOS, Life technologies). For better quality image overlay function was used [21, 27].

After seven days some of the seedlings in which the presence of N4T was validated by fluorescence microscope were transferred to a pot comprised of sterilized soil and allowed to grow further to verify the stable colonization of N4T inside the host. The pots were kept in the greenhouse (Alice Biotech, India) and regularly sterile distilled water was poured. After four weeks, the plants were taken out of the pot. The plants were first rinsed several times with sterile distilled water until the soil was removed. Then the plants were surface disinfected by the earlier-mentioned protocol [21]. After that, the roots and shoots were separated and transverse sections of root and shoot were prepared. The sections were then visualized under a fluorescence microscope (10X objective) for the presence of green fluorescence. The overlay function was used to attain a clear observation and the pictures were saved. Control plants not inoculated by N4T were compared.

3.3.5 Co-localization study of N4T with F1C1 in tomato seedlings

A gentamycin-resistant mCherry tagged F1C1 (TRS1016) was recruited for this study [27]. Freshly grown cultures of TRS1016 and *gfp* tagged N4T were obtained and the cells were re-suspended in sterile distilled water. Both the bacteria were mixed using 1 ml of each culture. Then the seedlings were inoculated with the root inoculation method

as described earlier. Both the tagged bacteria were also inoculated individually in tomato seedlings. The seedlings were kept in a controlled environment at 28 ºC temperatures, 80% Relative Humidity, and a photoperiod of 12 h. After the day of inoculation, a few seedlings were microscopically inspected for the colonization of N4T and F1C1 inside the seedlings. The seedlings were surface disinfected as described by Kumar et al. (2013), prior to the microscopic observation. The seedlings were directly visualized under a fluorescence microscope (EVOS, Life technologies). For better quality image overlay function was used.

3.3.6 Bio-control efficacy of N4T against F1C1 infection in grown up tomato plants

The efficacy of N4T in control of wilt disease caused by F1C1 was earlier studied in seedling stages of tomato by root and leaf inoculation. It was established that N4T could reduce the disease in tomato seedlings. To further study the efficacy of N4T in the control of F1C1 infection a study in grown-up plants was performed.

Seven days old tomato seedlings grown in a controlled environment as described earlier were recruited for this study. The seedlings were then transferred to the pot containing soil. In each pot, one seedling was kept and allowed to grow in a greenhouse. The seedlings were observed regularly and at regular time intervals, water was poured. Once the seedlings were grown to a certain level (4 weeks post transfer to pot), they were inoculated with bacterial suspension for bio-control study. For this study N4T and F1C1 cultures were allowed to grow in the BG medium and the concentration of both were adjusted to $\sim 10^8$ CFU/ml. The bacterial cells were resuspended in equal volumes of water. Four different inoculations were conducted 1. Plants were inoculated with only F1C1, 2. Plants were inoculated with N4T 3. Plants were inoculated with first N4T and then an equal volume of F1C1, 4. Only water was inoculated. For inoculation, small holes were made in the soil near the roots, and then bacterial cultures were poured. For each set mentioned above 5 plants were taken. For set one, 10 ml of F1C1 along with 10 ml of sterile distilled water was poured near the rhizosphere soils of each plant; for set 2, 10 ml of N4T and 10 ml of sterile distilled water was poured in each plant; for set 3, 10 ml of N4T and then 10 ml of F1C1 was poured in each plant; for set 4, 20ml of sterile distilled water was poured in each plant. The plants were kept in the greenhouse and at regular intervals, water was sprinkled in each pot. The plants were observed regularly for disease symptoms appearance.

3.3.7 Study of N4T colonization in brinjal seedlings

It was established by Phukan et al that *R. solanacearum* F1C1 can colonize inside brinjal seedlings as well they can cause disease in brinjal seedlings [29]. We were interested to find out if the tomato seedling isolates N4T strain can colonize the brinjal seedlings or not. To study N4T colonization, Dev Kiran cultivar of brinjal seeds were recruited and were allowed to grow till a two-cotyledon seedlings stage as mentioned above. The seedlings were surface sterilized as already mentioned and then TPP11; the GFP tagged strain of N4T was inoculated in the seedlings by root inoculation method and were incubated in a plant growth chamber at 28^{o} C, 80% Relative Humidity and a photoperiod of 12 h. Some of the seedlings were taken out after 3 days, surface sterilized as mentioned by Kumar *et al.*, 2013 and then observed under a fluorescence microscope using GFP filter. For better visualization overlay function was used. The seedling inoculated with wild-type N4T was taken as control.

3.3.8 Bio-control efficacy of N4T against F1C1 infection in brinjal seedlings

Once it was established that N4T can colonize in brinjal seedlings, it was studied for bio-control efficacy against F1C1 infection. It has already been documented that F1C1 can cause disease in brinjal seedlings grown under gnotobiotic conditions [29]. To study the bio-control efficacy, the brinjal seedlings were inoculated with F1C1 culture by leaf clip inoculation and were compared with brinjal seedlings inoculated with a mixed culture of F1C1 and N4T. For this work, the Dev Kiran cultivar of brinjal seeds was recruited and the seedlings were germinated as mentioned earlier. The two-cotyledon stage brinjal seedlings were selected in this study. Overnight-grown fresh cultures of F1C1 and N4T were taken for the study. F1C1 was mixed with different volumes of N4T and inoculated in brinjal seedlings by leaf inoculation method [29, 32]. The treatment sets included (1) F1C1+ N4T mix inoculation at a volume ratio of 1:1, (2) F1C1+ N4T mix inoculation at a 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 a negative control. After the experiment was performed repeatedly, the F1C1 and endophytes volume ratio were maintained at 1:50 for further biocontrol assays. A total of 60 seedlings were taken for each set of inoculation.

3.3.9 Hypersensitivity response test of *P. putida* N4T

The *P. putida* N4T strain was studied for hypersensitive response in tobacco plants that were recruited from Prof. L. Sahoo, IIT Ghy laboratory. It is already established that the phytopathogen *R. solanacearum* exhibits a hypersensitive response in tobacco, in this study, the F1C1 strain of *R. solanacearum* was recruited for comparison of hypersensitive response with N4T. Fresh-grown cultures of F1C1 and N4T in BG medium were taken for the study. Both cultures were infiltrated into the tobacco leaves by using a sterile syringe. While F1C1 was infiltrated twice in a different position, N4T was infiltrated once and sterile distilled water was infiltrated as control. The plant was monitored for the occurrence of hypersensitive response [27].

3.3.10 Creation of *gacA* mutant of *P. putida* N4T

Taking the reference genome of *P. Putida* BIRD-1 from the *Pseudomonas* genome database (http://www.pseudomonas.com), primers were designed for partial amplification of *gacA* homolog in the *P. Putida* N4T strain. The primers designed were incorporated with *Hind*III restriction sites in forward primer and an XbaI restriction site in the reverse primer at their 5' end respectively. These given Primers (5'-GCCAAGCTTGTGGTGGGTGAGGGAGATT-3';5''GCCTCTAGACAACGTCAGTTCAACGTCG-3') were employed for amplification of ~500 bp size amplicon of *gacA* gene homolog in N4T. Further, this amplicon was also sequenced for confirming homology with the *gacA* gene sequences of the BIRD-1 strain. For the cloning, the amplicon of *gacA* was ligated into a T-A cloning vector pTZ57R/T (Thermo Fisher Scientific) to get a construct pPLST1 (pTZ57R/T::$gacA_{N4T}$) by following the instructions of the manufacturing company. Then pPLST1 was subjected to restriction digestion with *Hin*dIII and *Xba*I enzymes simultaneously. The resulting ~500 bp amplicon from the previous step of digestion was gel-extracted and subsequently ligated to pCZ367 vector that harbors a promoter less *lacZ* reporter gene as well as ampicillin and gentamycin selection markers. But prior to ligation set-up, the pCZ367 vector was linearized with the help of the same pair of restriction enzymes, used in the digestion of the amplicon. Recombinant plasmid vector pPLST001 (pCZ367::$gacA_{N4T}$) was isolated from transformed DH5α strain of *E. coli* cells followed by confirmation of cloning step with the digestion of pPLST001 by the *Hin*dIII and *Xba*I enzymes.

After successfully generating the recombinant vector construct pPLST001, it was transformed in the *P. putida* N4T strain. Competent cells of N4T were prepared using $CaCl_2$ as mentioned above. For transformation, the aliquots of competent N4T cells were mixed with recombinant vector constructpPLST001 and incubated in ice for 30 minutes. The cell-DNA mixture was then subjected to heat shock at 42°C for 3 minutes and chilled in ice for 2 minutes and 30 seconds. After that 900 µl of BG broth was added to the mixture and the cells were allowed to incubate for one and half hours at 28°C in a shaking incubator. After a short spin, the supernatant was discarded and around 100µl of the cell pellets were spread on a BG plate containing ampicillin and gentamycin. Insertion mutation in g*acA* gene of successful transformant was confirmed by polymerase chain reaction. One insertion mutant TPP10 was recruited for subsequent experiments.

3.3.11 Antibacterial activity of *gacA* mutant of *P. putida* N4T against F1C1 and C10

In previous study, it was found that *P. putida* N4T could inhibit the bacterial phytopathogen *R. solanacearum* F1C1 in vitro and also could reduce the bacterial wilt disease caused by F1C1 in tomato seedlings (Under communication). Apart from inhibiting F1C1, N4T was also found to be exhibiting antagonistic activity against a bacterial endophyte isolated from tomato seedlings namely C10 which belongs to the *Enterobacter* species. C10 was also found to be inhibiting the pathogen F1C1 in vitro and was able to reduce bacterial wilt disease in tomato seedlings. As N4T could inhibit both F1C1 and C10, the *gacA* mutant strain of N4T (TPP10) was recruited and antibacterial activity was studied using an agar well diffusion assay. The bacterial culture of *R. solanacearum* F1C1, *P. putida*N4T, *Enterobacter* C10, and the *gacA* mutant strain of N4T was grown in BG medium overnight (OD_{600} ~0.5 for all). To study the inhibition of F1C1 by *gacA* mutant strain (TPP10), 100µl F1C1 was spread on BG agar plate and allowed to settle down for 30 minutes. A well was made in the center of the plate, and then 30µl of the *gacA* mutant was added to the center and the plate was kept at 28°C for 3 to 4 days. Similarly, F1C1 was tested against the wild-type N4T, which was already found to be inhibiting F1C1.To study the interaction of C10 with *gacA* mutant N4T, 100 µl of C10 was spread on BG agar plate and allowed to settle down for 30 minutes. A well was made in the center of the plate and 30 µl of the *gacA* mutant was added and the plate was kept at 28°C for 3 to 4 days. Likewise, the C10 was tested against wild-type N4T.

3.3.12 Inhibition of C10 by N4T in the presence of F1C1

Previously it was established in the laboratory that the pathogen F1C1 was inhibited by two bacterial isolates from tomato seedlings namely N4T and C10 (Unpublished work). Interestingly it was also observed that N4T could inhibit C10 in vitro. As N4T inhibits both C10 and F1C1 and C10 inhibits F1C1 a study was performed to understand the inhibition of C10 in the presence of F1C1. First, the C10 was spread in BG agar plate and N4T was added to the well and was kept at 28°C. The plate was observed time to time and after a certain point, a clear zone of inhibition appeared. Then after 36 hours, the plate was taken and on one side of the clear zone F1C1 was streaked and on the other side, C10 was streaked. And the plate was then again kept at 28 °C for a few days and observed at regular intervals to find out any changes in the appearance of the clear zone.

3.4 Results

3.4 .1 *P. putida*N4T can colonize the internal tissues of tomato

N4T was tagged with GFP to study the colonization in tomato seedlings. The plasmid, pDSK-GFPuv carrying one copy of *gfp* was successfully transformed in the N4T by using $Cacl_2$ treatment followed by a modified heat shock transformation method. The transformants colonies were observed under a fluorescence microscope using GFP filter. The appearance of green fluorescence confirmed the successful transformation of the plasmid. Also, the transformant colonies were studied against F1C1 for antagonistic activity in vitro. The colonies that were able to inhibit F1C1 were selected as successful transformants. One successful transformant TPP11 was taken for further study. The wild type N4T was belonging to *Pseudomonas putida*, TPP11 was also confirmed by performing 16S rDNA sequencing. The TPP11 strain was inoculated in 6 to 7 days old tomato seedlings by root inoculation. The seedlings were surface sterilized and observed under fluorescence microscopy. The seedlings inoculated with wild-type N4T were taken as control.

The presence of N4T was confirmed first in the second DPI in the root tip and root. And then the presence of N4T in the shoot was also observed. This eventually confirmed that N4T is indeed an endophyte. The cross-sections of the tomato seedlings and grown-up tomatoes inoculated by GFP-tagged N4T were examined under a fluorescence microscope. Green fluorescence was evident in the vascular tissues near the xylem of

both in tomato seedlings and grown-up tomato plants. This was interesting because *R. solanacearum* was already known for its ability to colonize in the xylem vessels.

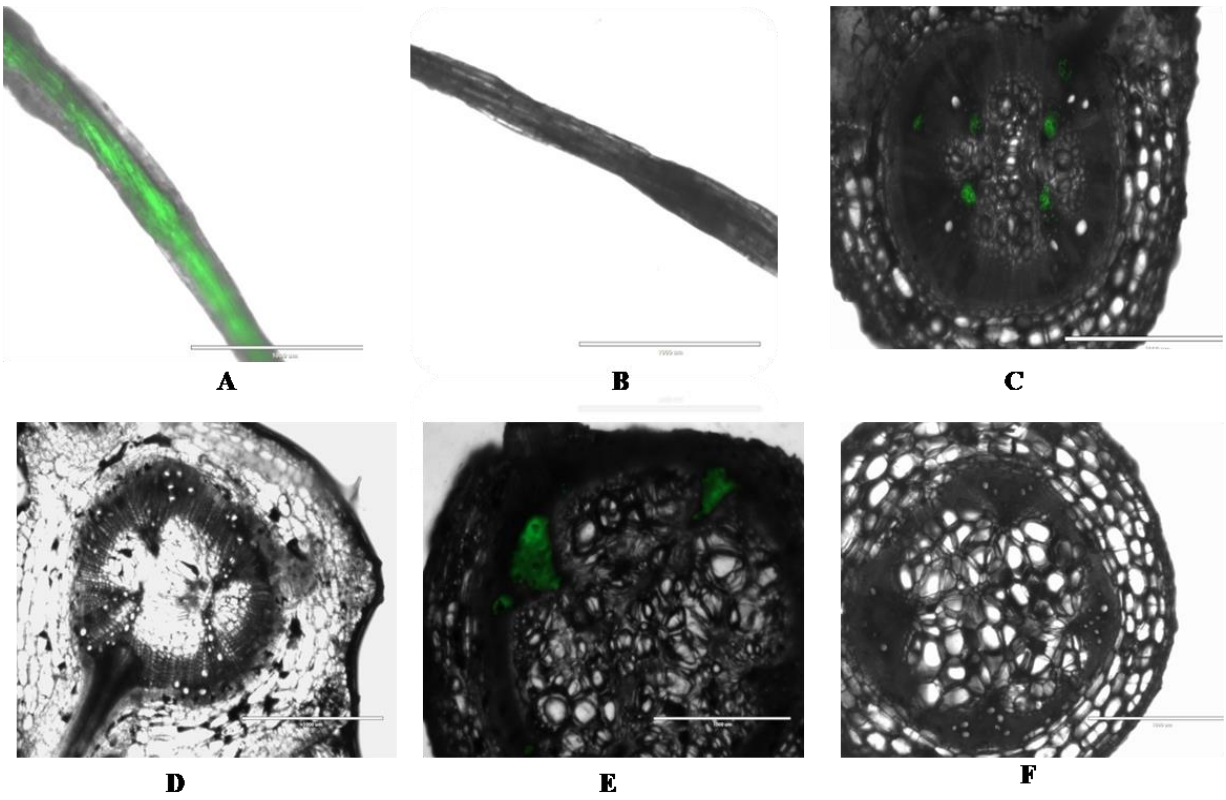

Fig 4.1: N4T colonization inside tomato seedlings and grown-up plants. A. N4T colonization in the root of tomato seedling at second DPI, B. Root of control seedling, C. Cross-section of the root of grown-up plant showing Presence of N4T in xylem, D. Cross-section of control grown-up plant root E. E. Cross-section of the stem of GFP N4T inoculated grown-up plants, F. Cross- section of the stem of control grown-up plant.

Further, a co-localization study was performed by recruiting a gentamycin-resistant mCherry tagged F1C1 (TRS1016) and *P. putida* N4T tagged with g*fp* (TPP11). Fresh-grown culture of both the strain was mixed in 1:1 ratio and was inoculated in tomato seedlings by root inoculation method. The seedlings were observed under the fluorescence microscope.

From the images of fluorescence microscopy, it was observed that both the N4T and F1C1 strain was localized in close proximity to the seedlings. Further detailed

investigation of the cross-sections of the seedlings and even in grown-up plants might give a clear indication of the co-localization of both the strains inside the host.

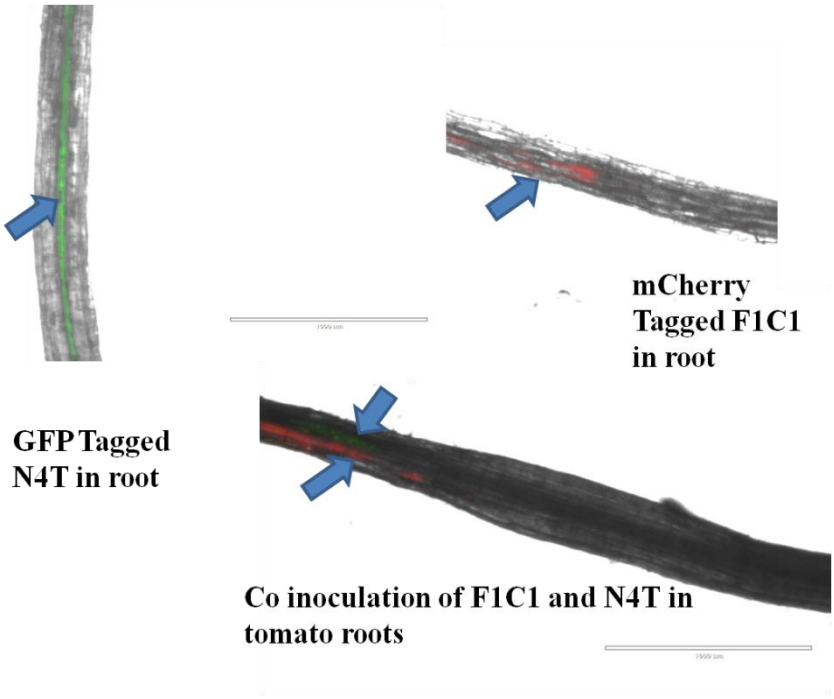

Fig 4.2: The fluorescence microscopy images of tomato seedlings showing the presence of both green and red fluorescence in the seedling that is inoculated by both N4T and F1C1. Only F1C1 and N4T inoculated seedlings were taken as control.

3.4.2 *P. putida* N4T could reduce the symptoms of bacterial wilt caused by *R. solanacearum* F1C1 in grown-up plants

The efficacy of N4T in the protection of against bacterial wilt disease caused by *R. solanacearum* F1C1 was examined in a greenhouse study. The N4T and F1C1 strains were inoculated by soil –the drench method. It was observed that only 2 plants were showing bacterial wilt symptoms those were inoculated by F1C1 and N4T, but all five plants were showing bacterial wilt symptoms that were inoculated by only the pathogen F1C1. While the plants inoculated by only N4T and the control plants where only sterile distilled water was inoculated were healthy. It was also observed that few plants that were inoculated with F1C1 showed symptoms earlier than those were inoculated with both F1C1 and N4T. In case of plants inoculated by F1C1 the earliest symptoms were observed on DPI 4 but the plants inoculated by N4T and F1C1 showed earliest symptoms in day 6. Further all the five plants inoculated by F1C1 were died by DPI 12 and 2 plants inoculated by both N4T and F1C1 were died by DPI 15.

This study suggested that N4T can reduce the bacterial wilt disease in grown up plants also the appearance of symptoms was also delayed in presence of N4T. This study needs to further carried out for clear verification of protection of the grown-up plants by N4T against F1C1 infection.

F1C1+N4T **F1C1** **N4T** **Control**

Fig 4.3: From left the first two pots are healthy plants inoculated by F1C1+N4T, the middle pot is a wilted plant infected by F1C1, the 4th pot from the left is a plant inoculated by N4T and the pot at the extreme right is plant inoculated with sterile distilled water.

3.4.3 *P. putida* N4T can colonize brinjal seedlings as well

In an earlier study, it was established that N4T can colonize tomato seedlings and grown-up plants. As *R. solanacearum* F1C1 can also infect brinjal seedlings we were interested to study if N4T can colonize the brinjal seedlings. The idea behind this study is to further investigate if N4T strain can be useful in the protection of brinjal seedlings against F1C1 infection.

For the colonization study, two cotyledon stage brinjal seedlings were recruited and GFP tagged strain of N4T was inoculated by root inoculation method as described earlier. The brinjal seedlings were taken out after each DPI; the surface sterilized and was observed under fluorescence microscopy as described above. The seedlings inoculated with the wild-type N4T (Not tagged with GFP) were taken as control.

Green fluorescence was observed in the root and stem of the seedlings inoculated by the tagged N4T and this established that N4T which was isolated from tomato seedlings could colonize the brinjal seedlings as well. This further stimulated us to perform an in

vivo study to examine the potential of N4T in the protection of brinjal seedlings against F1C1 infection.

3.4.4 Efficacy of *P. putida* N4T in protection of brinjal seedlings against F1C1 infection

Once it was established that N4T strain can colonize the brinjal seedlings the strain was further studied for its efficacy in controlling the disease caused by F1C1 in brinjal seedlings. The two-cotyledon stage brinjal seedlings were inoculated by mixed culture of the pathogen F1C1 and the bacterial endophyte N4T through leaf clip inoculation. From the experiment, it was observed that when F1C1 was mixed N4T in 1:50 ratio maximum protection was attained. When F1C1 was mix inoculated with water in 1:50 ratio, only 1 seedling was survived, on the other hand when F1C1 was mix inoculated with N4T in 1:50 ratio, 38 of 60 seedlings were survived, the infection was reduced by 60%. This study indicated that the tomato seedling isolate N4T was effective in controlling the disease caused by F1C1 in brinjal seedlings as well.

Fig 4.4: In vivo bio-control assay of brinjal seedlings: The picture on the left showing seedlings inoculated by F1C1. Only one seedling was survived, rests of them were killed by F1C1 infection. The picture on the right showing the seedlings inoculated with F1C1 and N4T mix culture (1:50).

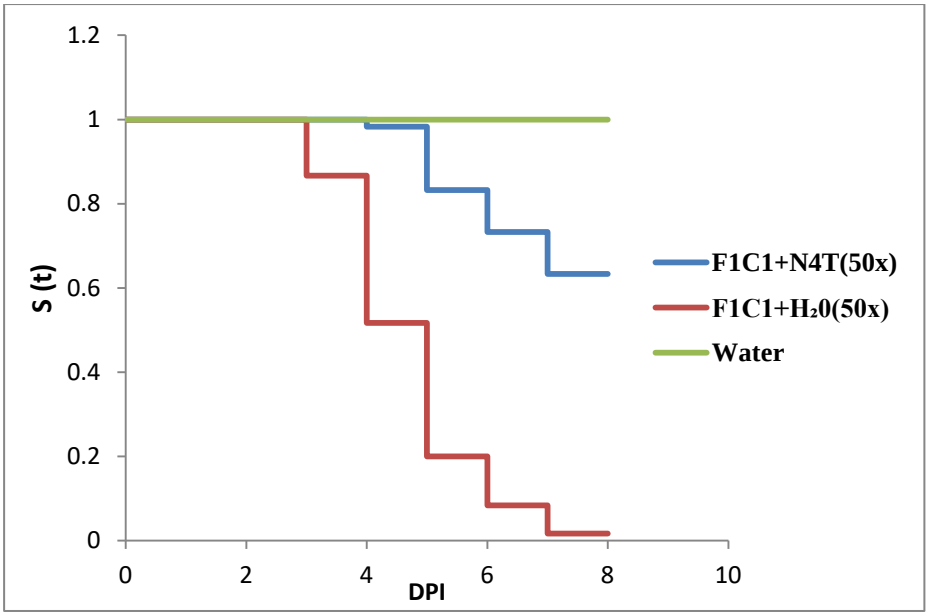

Fig 4.5: Kaplan-Meier survival analysis curve shows N4T could protect Eggplant seedlings when mix inoculated with F1C1. (p value<0.05)

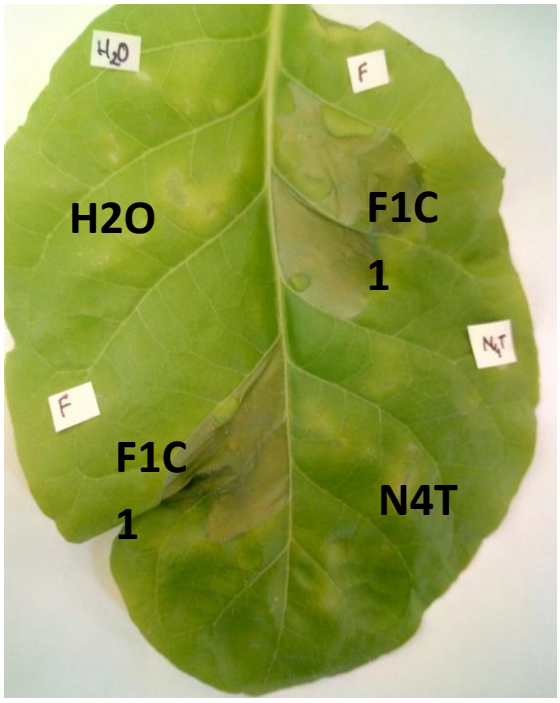

Fig 4.6: F1C1 showing hypersensitive response while N4T was found to be not showing significant hypersensitive response

3.4.5 *gacA* gene of *P. putida* N4T plays a role in the inhibition of C10 but not in F1C1

The *gacA* gene homolog was amplified using gene-specific primers taking the reference of the *P. putida* BIRD-1 genome. The amplicon was sequenced and confirmed. The genome sequence of N4T also revealed the presence of *gacA* gene homolog in it (under communication). The insertion mutation in *gacA* homolog was achieved by a single cross-over event using the plasmid pPLST001 (Table 1). $CaCl_2$ treatment followed by heat shock transformation of the N4T with the vector pPLST001 yielded transformants that were selected on BG-agar plates supplemented with ampicillin and gentamicin antibiotics. One of the successful transformants was taken for further study. Confirmation of the insertion of pPLST001 in *gacA* homolog was performed by PCR.

It was established that the wild-type N4T exhibits antagonistic activity against the pathogen F1C1 and bacterial endophyte C10. Therefore in this study, the mutant N4T strain was studied against F1C1 and C10 to understand if *gacA* plays any role in antagonism. For this agar well diffusion method was used as described in materials and methods. The antagonistic activity of the mutant strain of N4T was compared with the wild-type strain of N4T. It was found that the *gacA* mutant strain TPP001 was able to inhibit the F1C1 in vitro similarly to that of the wild-type N4T strain. This suggested that the *gacA* gene does not play any role in the inhibition of F1C1. On the contrary, while the wild-type N4T was able to inhibit C10 significantly, the *gacA* mutant was not showing significant inhibition in comparison to the wild-type. This suggests that *gacA* plays a role in the inhibition of C10. As the *gacA* is a response regulator and is believed to regulate many genes it will be interesting to find out which genes are involved in the antagonistic activity against C10. The results suggested that N4t employs different mechanisms to inhibit F1C1 and C10.

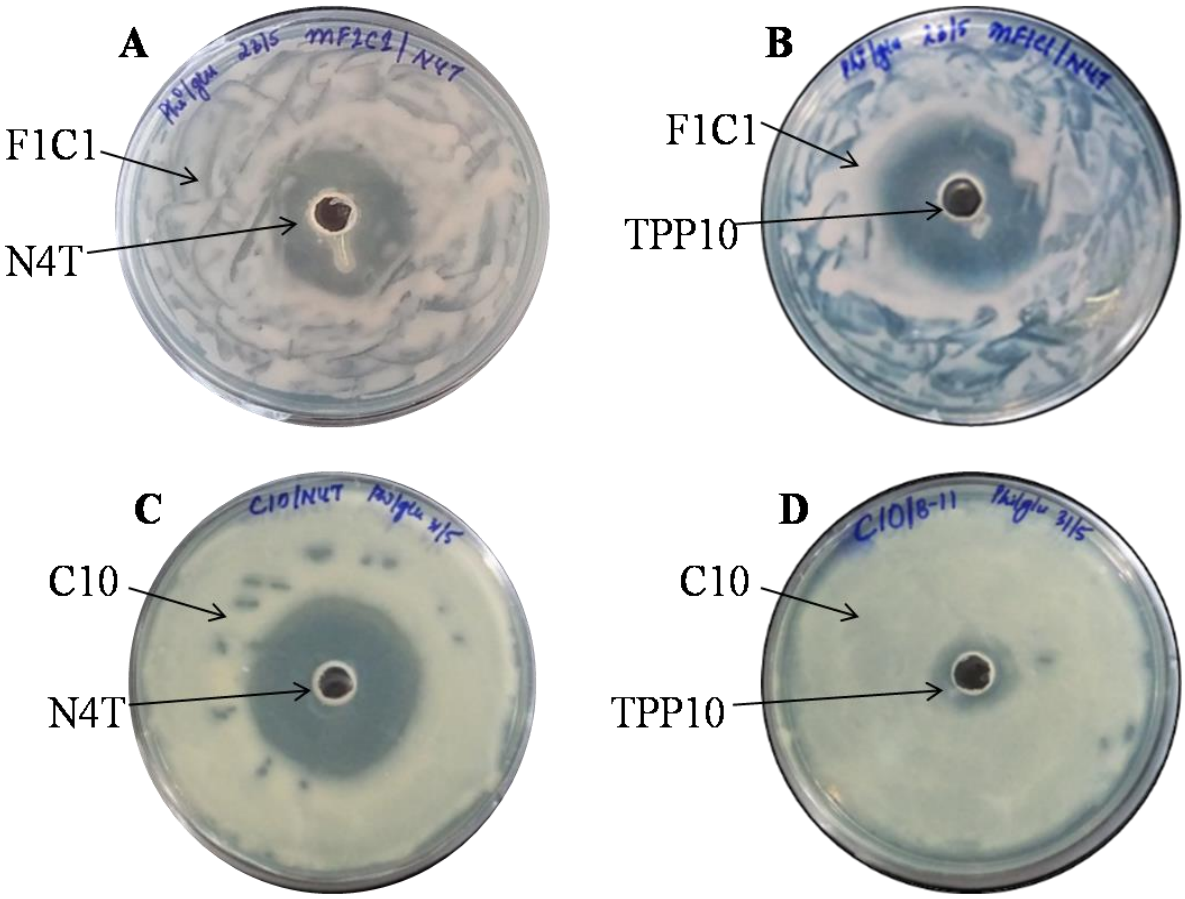

Fig 4.7: Study the inhibition of C10 and F1C1 by *gacA* mutant N4T strain (TPP001) by agar well diffusion method. A. N4T inhibiting F1C1, B. The *gacA* mutant inhibiting F1C1, C. N4T inhibiting C10, D. The inhibition study of C10 by the *gacA* mutant N4T, no significant clear zone was observed in compared to C.

3.4.6 The inhibition of C10 by N4T was increased in presence of F1C1

It was already established in the author's laboratory that N4T exhibits antagonistic activity against F1C1 and C10. A study was conducted to find out if the presence of F1C1 plays any role in the inhibition or not. First, the C10 was spread in BG agar plate and then wild type N4T was inoculated in the well. The plate was kept at 28°C and was observed at regular intervals. After 16 hours zone of inhibition was prominently observed. The zone was increased when observed in 24 hours. But the zone of inhibition did not increase in 36 hours, after 36 hours in one side of the clear zone F1C1 was streaked on the other side C10 was streaked and was kept at 28°C. The plate was observed at regular interval and it was interesting to find out that the clear zone was significantly increased when C10 and F1C1 were streaked; it seems that the presence of F1C1 might induce the inhibition of C10.As the mode of inhibition of C10 and F1C1 by

N4T were believed to be different, it will be interesting to find out the intricacy of the interactions of all three in the same environment. It is still unclear how the presence of F1C1 enhances the inhibition of C10. Further investigations are required to find out the mechanism of the interaction of all three when presenting together and also the mechanism of interaction with each other.

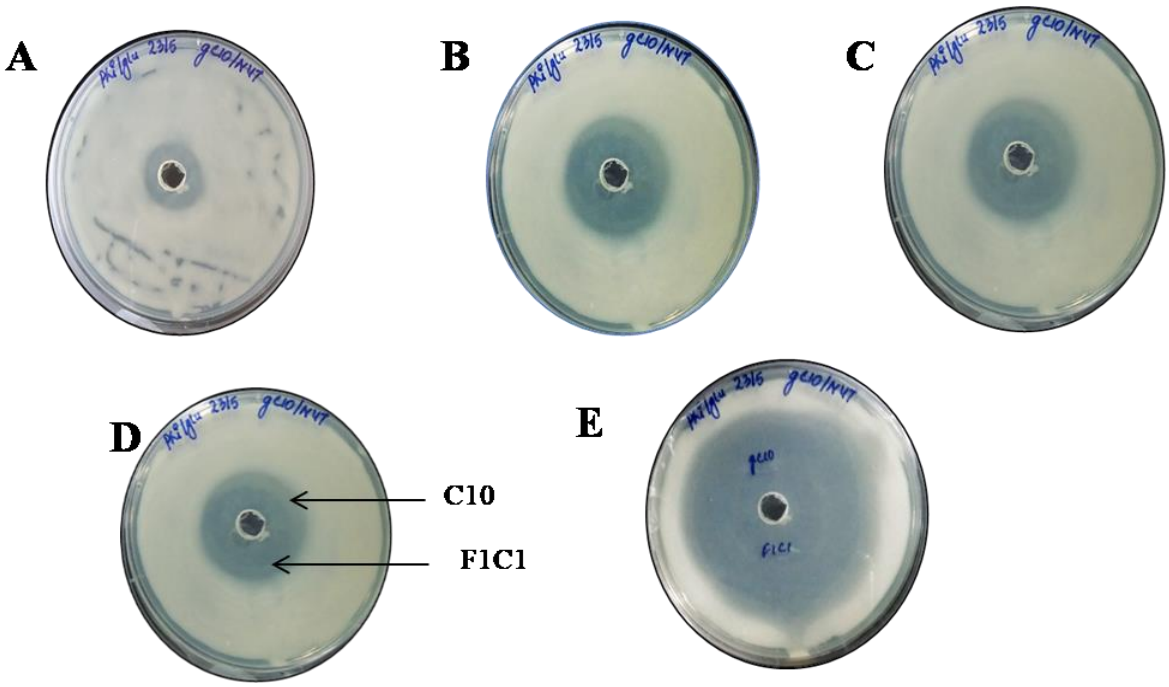

Fig 4.8: Inhibition of C10 in presence of F1C1, C10 was spread and N4T was added in the well A. Presence of inhibition zone in 16hours, B. Zone of inhibition in 24 hours, C. Zone of inhibition in 36 hours, D. C10 was streaked in one side of the zone of inhibition and F1C1 was streaked in the other side of zone of inhibition at 36 hours, E. Increased zone of inhibition in 8 days.

3.5 Discussion

Here in this work, we are documenting the detailed characterization of *P. putida* N4T, a *R. solanacearum* F1C1 antagonistic bacteria isolated from healthy tomato seedlings grown under gnotobiotic condition. Twenty-one bacteria were isolated from healthy tomato seedlings grown in soil free condition in a controlled environment and five of them were found to be inhibiting F1C1 growth in vitro which has been described in the previous chapter. Further all the twenty-one isolates were characterized and the five antagonistic bacteria were found to be effective in reducing the disease caused by F1C1 in tomato seedlings. In this study one F1C1 antagonistic isolate *P. putida* N4T was

investigated for different attributes. First, the bacterium was studied for its colonization ability in tomato seedlings and grown-up tomato plants. For this, a GFP tagged strain of N4T (TPP11) was created and inoculated in tomato seedlings and grown-up tomato plants. It was confirmed that N4T can colonize the internal tissue of tomato seedlings as well grown-up plants as the bacterium was found to be colonizing in the vascular tissue; this confirmed that N4T is indeed an endophyte. N4T was found to be effective in controlling the disease caused by F1C1 in tomato seedlings which is described in the previous chapter. To study the efficacy of N4T in controlling the disease caused by F1C1 in grown up tomato plants was studied in this chapter. The results indicated that N4T can reduce the disease and the appearance of symptoms was delayed in presence of N4T. A further experimental investigation is required for validation of the bio-control potential of N4T against F1C1 infection in grown up plants.

It is known that among many other plants, *R. solanacearum* causes bacterial wilt disease in brinjal also. The laboratory established previously that F1C1 strain can cause disease in brinjal seedlings also. As N4T was effective in controlling the disease caused by F1C1 in tomato, it was further tested in brinjal seedlings also. First it was confirmed that tomato seedling isolate N4T can also colonizes the brinjal seedlings and then a bio – control assay was carried out, which indicated that N4T strain was effective against F1C1 infection in brinjal seedlings too.

Apart from inhibiting F1C1, the N4T strain showed significant antagonistic activity against another bacterial isolate *Enterobacter* C10. The C10 strain on the other hand could also inhibit F1C1. In this study, the role of a gene *gacA* of *P. putida* N4T in the inhibition of F1C1 and C10 was inspected. The *gacA* gene in various strains of *Pseudomonas* spp. has been studied for its role in different biological functions. In this study an insertion mutation in *gacA* homolog of N4T was created by $Cacl_2$ treatment followed by heat shock method of chemical transformation. The *gacA* mutant was studied against F1C1 and C10 for antagonistic activity and also compared with the wild type N4T. It was found that the *gacA* mutant was exhibiting antagonistic activity against F1C1 in vitro like the wild type N4T, suggesting that *gacA* does not play role in antagonistic activity against F1C1. However, it was found that the *gacA* mutant was deficient in inhibiting C10 in comparison to wild type N4T in vitro. This implies that *gacA* plays role in inhibiting C10. This is interesting to observe that a single bacterium uses different mechanisms to inhibit different microorganisms. Further investigation is

required to fully understand the detailed mechanism of inhibition against both the bacteria by N4T. The *gacA* mutant strain of N4T was further studied in tomato seedlings for bio-control potential against F1C1 using the root dip inoculation method. In preliminary results, we observed that like the wild type N4T the *gacA* mutant was also able to reduce the occurrence of wilt disease in the tomato seedlings. The study has to be repeated for final confirmation in another study; we found that the inhibition of C10 by N4T increases further in the presence of F1C1. To know the detailed mechanism of what induces this extraordinary inhibition in-depth examination is required.

Considering the efficacy of the strain N4T to colonize inside two different hosts which are infected by *R. solanacearum* F1C1 and its ability to reduce the infection in both the hosts, it can be used as a potential bio-control agent in controlling bacterial wilt disease caused by *R. solanacearum* in future. Further investigation is required to unravel the mechanism of antagonistic activity of N4T against F1C1.

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