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Role of Rhizobacteria Associated with Diseased Tomato Plants towards their Response with *Ralstonia sp.*: The Bacterial Wilt Agent

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ABSTRACT

In this study, we have aimed to determine the antagonistic effects of various rhizobacteria against representatives of bacterial wilt disease; *Ralstonia pseudosolanacearum* with a comparison from control sterile distilled water (SDW) aliquots. During *in-vitro* study, bacterial wilt agent was clearly inhibited in their growth by significant percentage by 32 different strains through perforated agar plate method. Results showed 10 potential strains among those 32 strains viz. DIB76/BCS-8, DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-12, DIB76/BCS-19, DIB76/BCS-20, DIB76/BCS-21, DIB76/BCS-24, DIB76/BCS-26 and DIB76/BCS-27 had the highest growth suppression for 9 different strains of *R. pseudosolanacearum* as compared to other bacterial strains. Trials with tomato crop at polyhouse had no incidence of bacterial wilt than those for control. Study, thus identifies 10 rhizobacterial strains from rhizosphere soil of diseased tomato plant possessing potential antagonistic activity against the wilt pathogen and has prospects as good biocontrol agents.

Keywords: Antagonistic activity; Perforated agar plate method; Bio-control

1. INTRODUCTION

Researches from past many years have repetitively established that phylogenetically diverse micro-organisms may behave as natural antagonists to numerous phyto-pathogens. Evidences indicate the importance of rhamnolipid/siderophore/antibiotics in bio-control of phyto-pathogens[1-7]. The rhizosphere soil of healthy plants is usually regarded as a good source for isolating PGPRs and bio-control agents (BCAs)[8,9]. Since 1980, rhizobacteria have been investigated as a possible substitute of chemicals and are used to control a broad range of plant diseases either by antibiotics production, competition, predation, induction of host resistance, siderophore and/or most recently discovered bio-surfactants[10]. Antibiotics production by microorganism is considered as a major event in soil borne disease suppression ability of rhizobacteria. Variety of antibiotics and metabolites such as phenazine, carboxylic acid, pyoluterin, 2, 4-diacetyl phloroglucinol, oomycin and cyanide produced by microbes may also be responsible for control of certain phyto-pathogens. Plant pathogens are the most vital agents that cause maximum loss of agricultural produces every year. Farmers vividly use chemical pesticides and fungicides to minimise the losses but these chemicals have toxic effect on plant, soil and human health.

Bacterial wilts of tomato, potato, pepper, eggplant was among the first disease that was proved to be caused by bacteria by the scientist Smith. Bacterial wilt occur widely in tropical, subtropical and some temperate regions of the world[11] and is caused by a soil borne vascular pathogen; *Ralstonia solanacearum species complex*[12] which is later been classified into *Ralstonia pseudosolanacearum* based on phylotype characterisation. The research work published by Safini et.al. in 2014 has the view of inclusion of all the strains belonging to *Ralstonia solanacearum* phylotype I to a new species i.e. *Ralstonia pseudosolanacearum*[13]. The pathogen has host plants more than 450 plant species belonging to both monocots and dicots[14-17]. For the control of this pathogens, certain control methods such as crop rotation, field sanitisation, deploy of resistant varieties deliver partial success as it has wide spread geographic distribution, high survival properties and unusual wide host range. Chemical control method for this disease is generally ineffective and soil fumigation has negligible effect[18,19]. Till date there is no effective chemical control product commercially available for bacterial wilt. Disease resistance of a cultivar is usually not stable and/or durable[20,21]. Therefore, biological control is an alternative method to resolve some of these difficulties. This method also avoids environmental pollution. Several researches have been carried out to find bio-control agents to reduce bacterial wilt severity.

In India, certain research work has been reported on the bio-control of bacterial wilt disease. The work published by Singh[22], *et. al.* signifies the role of *Bacillus amyloliquifaciens* in bio-control of *Ralstonia solanacearum*. Another work by Maji et.al reported the significance of different strains of *Pseudomonas* in bio-control of bacterial wilt disease as well as plant growth promotion activity in tomato plants[23]. The research work done by Ramesh[24], *et. al.* concludes that, *Pseudomonas* is the major antagonistic endophytic bacteria from eggplants that have the potential to be used as a bio-control agent as well as plant growth-promoting rhizobacteria. The large scale field

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evaluation and detailed knowledge on antagonistic mechanism can provide an effective bio-control solution for bacterial wilt of solanaceous crops. So, in this need of the hour, the prerequisite of sustainable agriculture will rely on the incorporation of microbiology with conventional agricultural practices. The most sustainable and environmentally suitable control method may be accomplished by means of bio-control agents to reduce the use of agrochemicals and their toxic residues in the environment and in food stuff.

2. MATERIALS AND METHODS

This study was carried out at Defence Institute of Bio-energy Research, DRDO, Haldwani, Uttarakhand during February 2015 to May 2019. The study comprised of three parts:

(i) Isolation and multiplication of rhizobacteria
(ii) *In-vitro* screening for potential antagonists against different strains of *Ralstonia pseudosolanacearum* and
(iii) *In-vivo* study for the control of bacterial wilt diseases in tomato crops.

2.1 Isolation and Multiplication of Rhizobacteria

Rhizosphere soil samples from diseased tomato plants and diseased plant parts were collected from 12 different sites at different terrains of Uttarakhand. Soils and plants were tagged properly. Samples were kept in refrigerator (at 8 ^{0}C) in the laboratory. A representative subsample of 10 g (from each sampling sites) was taken after *Conning and Quartering* method[25] and standard microbiological isolation procedure was followed for isolation and purification of isolates[26]. The biocontrol strains viz. DIB76/BCS-8, DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-12, DIB76/BCS-19, DIB76/BCS-20, DIB76/BCS-21, DIB76/BCS-24, DIB76/BCS-26 and DIB76/BCS-27 have been used during this study.

2.2 *In-vitro* Screening for Potential Antagonists Against Strains of *Ralstonia pseudosolanacearum*

All the 98 bacterial strains were screened for their antagonistic behaviour towards 9 different strains of *R. pseudosolanacearum.* (3 strains obtained from NAIMCC and 6 DIBER isolates). All the R. *pseudosolanacearum* strains belong to phylotype I of *R. solanacearum species complex*[13]. For this, the pathogen strains were inoculated in SMSA broth & was maintained at 1.5×10^7 CFU/ml whereas the test bacterial strains were inoculated in Kings medium B base broth & Mueller Hinton broth individually and maintained at 1.8×10^8 CFU/ml. The test was performed through perforated agar plate method on Kings medium B base agar plates and Mueller Hinton agar plates following incubation at 28 ^{0}C for 48-72 h[27]. The strains showing considerable inhibition zones were taken further for next experiments.

Preparation of washed cell culture: Washed cell cultures (WCC) for both the pathogen strains viz. *R.s.*00418 and DIB-117 and the antagonist strains viz. DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 were prepared as per standard procedure. OD adjusted to 0.5 Mc Farland standard constant. The screened bacterial strains were again checked for their antagonistic activity as described earlier. The experiment was repeated at least 3 times on both the media plates.

2.3 In-vivo Study for the Control Of Bacterial Wilt Diseases in Tomato Crops

This experiment involved pot trials with tomato crop variety D-68 (susceptible for bacterial wilt disease) in 2 different methods: seed priming method and soil soaked method at polyhouse conditions.

2.3.1Seed Priming with Antagonist Strains

For this, washed cell cultures of 5 antagonist strains DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 were prepared and OD maintained at 1.8×10^8 CFU/ml. Tomato seed variety D-68 was taken and washed.

Seed washing protocol: 1 gm of tomato seeds taken. Suspended in 100% isopropanol for 45-60 s. Dipped in 50% bleach containing 0.05% tween 20 for 5 min. Rinsed with sterile distilled water for 7-8 times (until the smell of bleach resides). Seeds were then resuspended in sterile distilled water and kept for overnight.

Seed priming: washed tomato seed taken in petriplates and flooded with 0.2% CMC. Shed dried for 3-4 h. 10 ml WCC (containing 1.8×10^8 CFU/ml cells) of antagonist strains added to the seeds and kept overnight.

Next day primed seeds were transferred to sterile pots (9×10 cm) containing tyndalised soil mixture (soil: coccapit: vermiculite :: 2:1:1) and sowed. Seeds were germinated in around a week. Seedlings were allowed to grow upto 4-5 true leaves condition. Plants were challenge inoculated with 20 ml WCC (containing 1.5×10^7 CFU/ml cells) of 2 pathogen strains viz. R.s.00418 (culture obtained from NAIMCC) & DIB 117(DIBER isolate). For pathogen only control sets untreated tomato seeds were sowed and treated with pathogen strains only. Day to day observation for appearance of wilt in the leaves for 15 days was taken and disease severity was noted down in the form of disease index of scale 0-5: 0, no wilting/ healthy plant; 1, partial wilting of one lower leaf; 2, wilting of two or three lower leaves; 3, wilting in all leaves except top two or three leaves; 4, wilting of all the leaves; 5, plant dead. Disease severity was calculated using the formula:

$$DI = (5A+4B+3C+2D+E) / 5N \times 100$$

where A= no. of plants on scale 5; B= no. of plants on scale 4; C= no. of plants on scale 3; D= no. of plants on scale 2; E= no. of plants on scale 1; N= total no. of plants[28].

Experiment was repeated 3 times in 3 different seasons viz. Sept 2017 to Nov 2017, Feb 2018 to April 2018, Nov 2018 to Feb 2019. Each experiment contained 3 replicates (pots) per treatment and each pot contained 3 plants inoculated with pathogen strains. Plants were arranged in completely randomised design in a polyhouse.

2.3.2 Soil Soaked Method

For this experiment, tomato seeds variety D-68 was taken and washed as described earlier. Seeds were sowed in trays containing tyndalised soil mixture (soil: coccapit:

vermiculite:: 2:1:1) and seedlings were allowed to grow upto 3 weeks. Then 3 week old seedlings were transferred to sterile pots (9×10 cm) containing tyndalised soil mixture. 30ml WCC (containing 1.8×10^8 CFU/ml cells) of 5 antagonist strains poured individually to the pots containing tomato seedlings on the same day of transplantation. After 5 days of transplantation, the plants were challenge inoculated with 20 ml WCC (containing 1.5×10^7 CFU/ml cells) of pathogen strains R.s. 00418 & DIB 117. For control set of plants, 20 ml of sterile distilled water added to the pots. For pathogen only control sets, tomato plants were inoculated with 20 ml pathogen strains only. Day to day observation for appearance of wilt in the leaves for 15 days was taken and disease severity was noted down as described earlier.

3. RESULTS

3.1 Isolation of bacterial strains

From 12 different rhizosphere soil samples, isolation yield 98 bacterial isolates and 47 fungal isolates. Only bacterial strains were carried further for this study.

3.2 *In-vitro* Screening and Identification of Potential Antagonists

Among the 98 bacterial strains, 32 strains showed potential growth inhibition to at least 1 strain of *R. pseudosolanacearum*. Among these 32, the strains DIB76/BCS-8, DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-12, DIB76/BCS-19, DIB76/BCS-20, DIB76/BCS-21, DIB76/BCS-24, DIB76/BCS-26 and DIB76/BCS-27 displayed significant average inhibition zones of sizes 30mm, 24mm, 18mm, 22mm, 16mm, 18mm, 24mm, 22mm, 16mm and 26mm respectively. These strains were effective in all test methods. (Tables 1 and 2, Figs. 1 and 2).

3.2 Identification of Strains

Identification of potential strains before *in-vivo* experiment was necessary to check whether or the antagonist strain is a plant, animal or human pathogen. The pathogenic strains cannot be used in polyhouse conditions and can never be applied in field conditions due to ethical issues as these will adversely affect the surrounding environment and also public health. So, 16SrRNA sequencing of the potential strains was

Table 1. In-vitro antibiosis test results against *R. solanacearum* and *R. pseudosolanacearum* strains on Kings medium B base agar plates

Antagonist strains	Antibiosis test results (on Kings medium B base agar plates) against *R. pseudosolanacearum* strains (inhibition zone size in mm)								
	0418	SIK	IDK	DIB 115	DIB 116	DIB 117	DIB 118	DIB 119	DIB 120
DIB-76/BCS-8	27	No zone	19 (cz)	34	No zone	12	23	35	35
DIB-76/BCS-9	18	23	24	15	22	14	20	23	22
DIB-76/BCS-10	27	28	18	33	21	24	53	27	40
DIB-76/BCS-12	21	19	17	No zone	16 (cz)	18	36	16	15
DIB-76/BCS-19	30	17	No zone	No zone	18	No zone	No zone	14	18
DIB-76/BCS-20	16	22	No zone	No zone	19	No zone	No zone	14	19
DIB-76/BCS-21	18	22	18 (cz)	36	24	No zone	42	17 (cz)	24
DIB-76/BCS-24	20	23	21	28	19	12	14	12	16
DIB-76/BCS-26	19	No zone	22	No zone	27	No zone	No zone	14	33
DIB-76/BCS-27	18	28	24	16	26	21	15	16	42

Table 2. In-vitro antibiosis test on Mueller Hinton Agar plate against *R. pseudosolanacearum* strains

Antagonist strains	Antibiosis test results (on Mueller Hinton agar plates) against *R. pseudosolanacearum* strains (inhibition zone size in mm)								
	0418	SIK	IDK	DIB 115	DIB 116	DIB 117	DIB 118	DIB 119	DIB 120
DIB-76/BCS-8	32 (cz)	20	No zone	31	28	34	22 (cz)	22	24
DIB-76/BCS-9	21	25	26	30	29	34	51	20 (cz)	18
DIB-76/BCS-10	27	No zone	21	No zone	29	33	No zone	No zone	18
DIB-76/BCS-12	16	21	19	14	22	18	16 (cz)	18 (cz)	13 (cz)
DIB-76/BCS-19	28	No zone	No zone	No zone	23	16	19	No zone	14
DIB-76/BCS-20	31 (cz)	No zone	No zone	No zone	25	42	21	No zone	No zone
DIB-76/BCS-21	21	No zone	No zone	21 (cz)	30	19	19 (cz)	31 (cz)	32 (cz)
DIB-76/BCS-24	30	25	21	No zone	28	34	46 (cz)	26 (cz)	No zone
DIB-76/BCS-26	31	24	20	36	14	16	21	19 (cz)	No zone
DIB-76/BCS-27	34	27	24	30	41	24	42	30 (cz)	34 (cz)

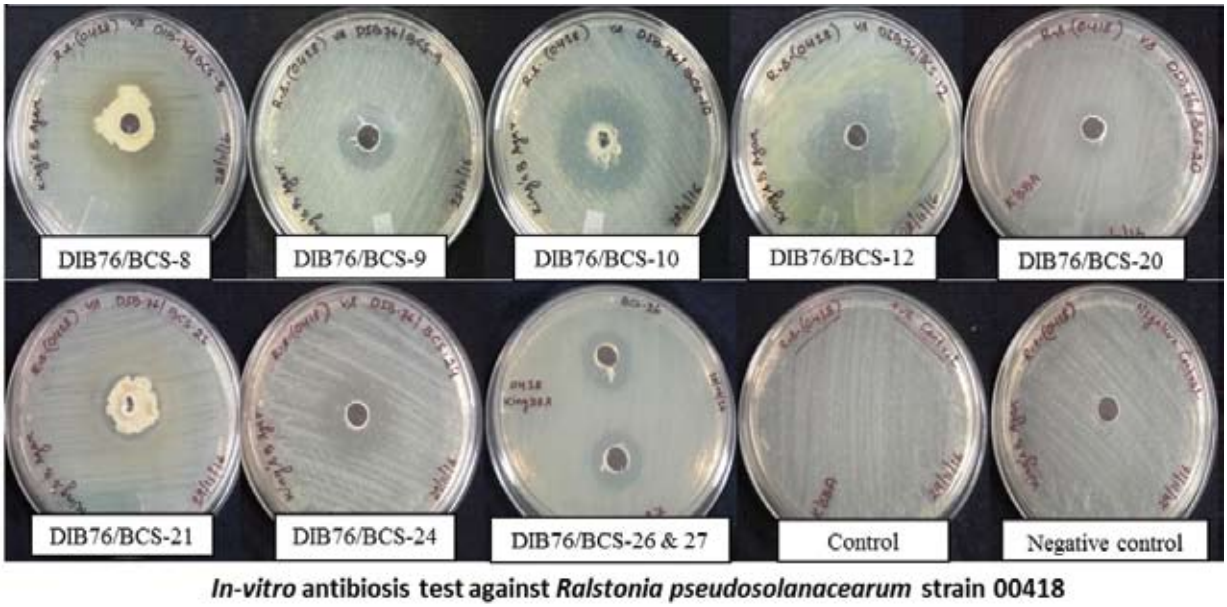

Figure 1. Representative images of *In-vitro* antibiosis test results against *R. pesudosolanacearum* strain on Kings medium B base agar plates.

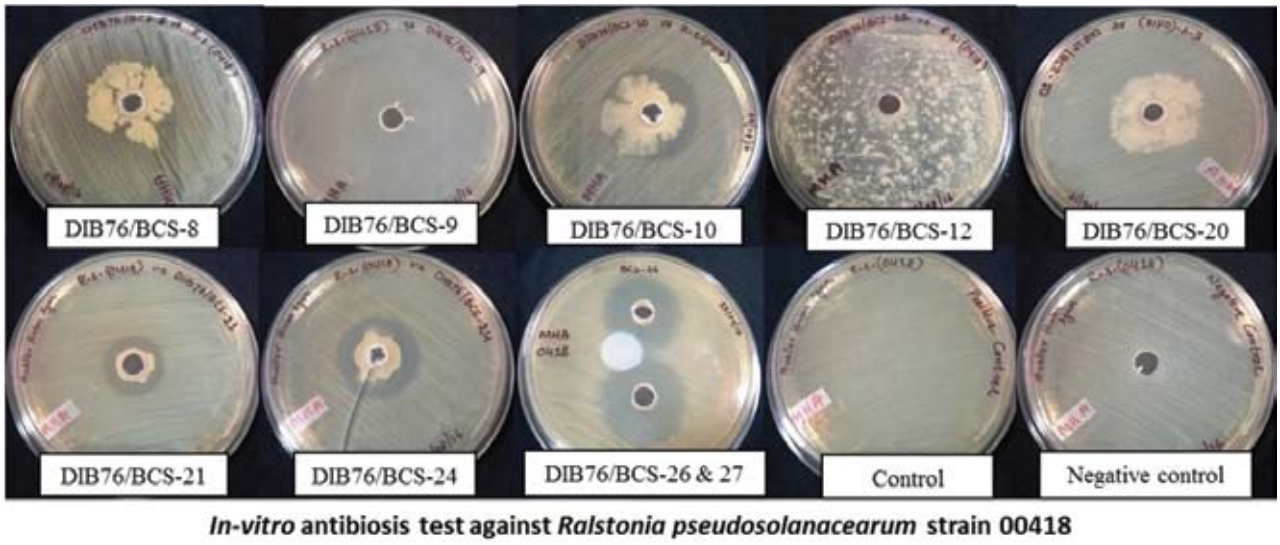

Figure 2. Representative images *In-vitro* antibiosis test results against *R. pseudosolanacearum* strain on Mueller Hinton agar plates.

done and are identified as follows. The strain sequences were submitted to NCBI data base and accession numbers have been assigned as shown in Table 3.

Identification revealed the strains were not pathogenic either to plant, animal or human and hence can be used further. The strains DIB76/BCS (10, 19, and 20) were the same species. So DIB76/BCS-10 strain was chosen for in-vivo study.

3.1 *In-vivo* Study Results

Only 5 different strains viz DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 were taken for this study. The study revealed, there was considerably low or no incidence of bacterial wilt disease in antagonist strain treated plants as compared to pathogen only control treated plants in polyhouse conditions. Results for disease index for all the set of plants are given in tabular form along with graphical representation and pictures as shown in Table 4, and Figs. 3-5.

Table 3. Identification of important biocontrol strains

Strain name	Identification	NCBI accession no.
DIB76/BCS 8	*Bacillus spp.*	MK801247
DIB76/BCS 9	*Stenotrophomonas spp.*	MK835671
DIB76/BCS 10	*Bacillus cereus*	MK840995
DIB76/BCS 12	*Paenibacillus alveri*	MK835674
DIB76/BCS 19	*Bacillus cereus*	MK840997
DIB76/BCS 20	*Bacillus cereus*	MK840998
DIB76/BCS 21	*Bacillus subtilis*	MK841040
DIB76/BCS 24	*Bacillus methylotrophicus*	Pending
DIB76/BCS 26	*Bacillus safensis*	MK841312
DIB76/BCS 27	*Brevibacillus laterosporus*	Pending

Table 4. In-vivo expt.: Disease index in treated and control plants

Treatments	Disease index (%)	
	Seed priming	Soil soaked
D-68+ DIB76/BCS-9+ 00418	0	0
D-68+ DIB76/BCS-10+00418	0	0
D-68+ DIB76/BCS-21+ 00418	0	0
D-68+ DIB76/BCS-24+ 00418	0	0
D-68+ DIB76/BCS-27+ 00418	0	0
Control (D-68+Pathogen only 00418)	100	26.6
D-68+ DIB76/BCS-9+DIB 117	0	0
D-68+ DIB76/BCS-10+DIB 117	0	0
D-68+ DIB76/BCS-21+DIB 117	0	0
D-68+ DIB76/BCS-24+DIB 117	0	0
D-68+ DIB76/BCS-27+DIB 117	0	0
Control (D-68+ Pathogen only DIB-117)	100	60

Figures 3-5; representative pictures of *in planta* experiment for appearance of wilt symptoms in 45 days old treated tomato plants along with control.

4. DISCUSSION

The aim of this study is to find out some suitable bio-control strains to combat bacterial wilt disease. To fulfil the objectives, we have isolated 98 strains from the rhizosphere soil of diseased tomato plants. The rhizosphere soil of healthy plants is usually regarded as a good source for isolating PGPRs and Bio-control Agents (BCAs)[8,9]. Growth inhibition of the pathogens may be caused by production of antibiotics by the bio-control micro-organisms, through nutrient competition, by siderophore production, by production of toxic substances like NH3 or HCN and/or by production of bio-surfactants. Antibiotics and secondary bio-metabolites production by microorganisms are considered as the major factors in soil borne disease suppression ability of rhizobacteria[29]. The possible suppression mechanisms of BCAs may also involve induced systemic resistance, antibiosis, the production of degrading enzymes that degrade the cell wall, production of substances like b-exotoxins, bacteriocins and a signal molecule in the bacterial quorum-sensing system[28,30-35] .

All the 98 isolates were evaluated *in-vitro* for their antagonistic behaviour by agar well diffusion method. Out of 98 strains, only 5 strains viz. DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 has selected for *in-vivo* studies based on their antibiotic retrieval properties as evidenced by prominent inhibition zones against various strains of *R. pseudosolanacearum* in *in-vitro* studies. Result of *in-vitro* studies could be generalised to real field conditions. Hence, *in-vivo* studies were performed in controlled environmental conditions inside polyhouse. The tomato variety used for *in-vivo* studies is prone to bacterial wilt disease as shown in Table 4. We preceded the *in-vivo* studies by two methods; seed priming and soil soak method by standard protocols. Seed coating method is performed for preventing infection whereas soil soak method activates the plant defence system. It has been reported that some *Bacillus* spp. strains produce elicitors that activate the plant defence system[36,37]. Studies with both the methods indicate that, the selected bio-control strains DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 were successful in preventing wilting in experimental tomato plants. Previous studies have indicated that antimicrobial substances produced by biocontrol agents are effective in eliminating the phytopathogens before host invasion. Studies also have indicated that elicitation of ISR by *B. subtilis* is associated with changes in cell wall composition, de novo production of pathogenesis-related (PR) proteins such as chitinases and glucanases, and synthesis of phytoalexins

Figure 3. Tomato plants treated with biocontrol strains along with pathogen strain *R.s.00418* via soil soaked method.

Figure 4. Tomato plants treated with biocontrol strains along with pathogen strain *DIB-117* via soil soaked method.

Figure 5. Pathogen only control in soil soak method and appearance of wilting.

associated with resistance. In most cases, multiple compounds generated by one *B. subtilis* strain are involved in the restraint of pathogens[38]. In general, vulnerability of the rhizosphere for invasion by soil pathogens is inversely associated to the diversity of the rhizosphere microbiome whereby amplified diversity can result in reduced pathogen virulence[39]. From our study, we are of the view that, the strains; DIB76/BCS-9, DIB76/BCS-10, DIB76/BCS-21, DIB76/BCS-24 and DIB76/BCS-27 produce certain antibacterial (may be antibiotics or enzymes) substances as evidenced in *in-vitro* studies. Hence these strains were successful in abolishing the pathogens thereby preventing pathogen invasion and the disease.

5. CONCLUSION

The disease prevention activity of these antagonist rhizobacterial strains against infection with *Ralstonia pseudosolanacearum* indicates that, these strains can be used as a microbial biocontrol agent to prevent plant diseases. Further, these studies provide a clue for the production of secondary bio-active compounds. This study thus indicates the potential of rhizobacteria as bi-functional bio-pesticide to control phytopathogens for plant protection. The disease deterrence activity and indication of subsequent activation of the plant defence system will contribute to further evaluation and practicality of these strains as effective biocontrol agent.

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In the current study, she has contributed in the critical revision and editing of the manuscript.

Appendix

➢ **Compositions of Media used in the study:**

Nutrient agar Media	**Gram/lit**
• Peptone	5
• Meat extract B	1.5
• Yeast extract	1.5
• Sodium chloride	5
• Agar	17

Nutrient Broth Media	**Gram/lit**
• Peptone	5
• Meat extract B	1.5
• Yeast extract	1.5
• Sodium chloride	5

Mueller Hinton agar Media	**Gram/lit**
• HM infusion B from	5
• Acicase	1.5
• Starch	1.5
• Agar	17

Mueller Hinton broth Media	**Gram/lit**
• HM infusion B from	5
• Acicase	1.5
• Starch	1.5

Kings medium B base agar	**Gram or Ml/lit**
• Proteose peptone	20
• K_2HPO_4	1.5
• $MgSO_4.7H_2O$	1.5
• Glycerol	15
• Agar	20

Kings medium B base broth	**Gram or Ml/lit**
• Proteose peptone	20
• K_2HPO_4	1.5
• $MgSO_4.7H_2O$	1.5
• Glycerol	15

SMSA agar Media	**Gram or Ml/lit**
• Bacto peptone	10
• Casamino acid hydrolysate	1
• Glycerol	5
• Agar	20

SMSA broth Media	**Gram or Ml/lit**
• Bacto peptone	10
• Casamino acid hydrolysate	1
• Glycerol	5