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Management of bacterial wilt of tomato caused by *Ralstonia solanacearum* using PGPRs and endophytic microbes

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Abstract
The study was made to assess the PGPR and endophytic microbial population of two different species of tomato plants [*Solanum pimpinellifolium* (kon bilahi) and *Solanum lycopericum*] and develop a biocontrol strategy using these microbes for managing bacterial wilt (BW) of tomato caused by *Ralstonia solanacearum*. In this study, we observed greater microbial diversity of PGPR and endophytic microbes in *S. pimpinellifolium* compared to *S. lycopericum*. By performing the *in-vitro* antagonistic and compatibility studies, it was revealed that a few promising PGPR (RKB7) and endophytic (EKA4 and EKB6) microbes could effectively inhibit the bacterial wilt pathogen. Combination of these isolates (RKB7+EKA4+EKB6) showed the highest inhibition of BW pathogen in tomato crops. Molecular characterization and nucleotide analysis revealed that RKB7, EKA4 and EKB6 isolates were found to be *Bacillus subtilis* (ON261568.1), *Streptomyces virginiae* (ON223266.1) *and Pseudomonas aeruginosa* (ON248938.1) respectively. Effective growth-promoting characteristics such as the production of ammonia, IAA, HCN, siderophore and zinc and phosphorus solubilisation were recorded for these isolates. Best PGPR and endophytic microbes and their combination along with an antibiotic check, streptocycline @ 200 ppm was tested *in vivo* for their efficacy in managing BW of tomato. The lowest disease incidence (31.11%) and highest yield (340.67 g/plant) were recorded in the plants treated with combination of these isolates (RKB7+EKA4+EKB6). Similarly, other yield attributing characters *viz.,* plant height, root length, no. of leaves, etc., were found to be high in the plants treated with the combination of the isolates.

Keywords: Endophyte, PGPR, tomato, bacterial wilt, *Ralstonia solanacearum*

Introduction

In the current era of agricultural research, the significance of sustainable agriculture is emerging as a vital issue. In this context, the application of endophytes and Plant growth promoting rhizobacteria (PGPR) has expanded dramatically over the last several decades in various parts of the world. Endophytes are microorganisms such as bacteria, fungi, archaea and protists that inhabit and colonize the interiors of plants (Hardoim *et al.,* 2015) [30]. These are known to promote host plant growth and antagonize pathogens due to its characteristics like ability to synthesize plant hormones, solubilize phosphate, secrete siderophores, etc. (Bastian *et al*., 1998; Gaiero *et al*., 2013; Kumar *et al*., 2015) [76, 24, 43]. Plant growth promoting rhizobacteria (PGPRs) are those bacteria which inhabit the plant rhizosphere. PGPRs through their direct or indirect mechanisms promote plant health by the production of phytohormones, siderophores, inducing plant systemic resistance responses, interfering in the bacterial quorum sensing (QS) systems, etc. (Ahmad *et al.* 2016) [77].

India's northeast region (NER) is recognised as one of Asia's biodiversity hotspots. It supports a variety of unexplored microbial populations due to diverse variations in climate, terrain, soil properties, and natural resources. Therefore, the exploitation of these PGPRs and endophytic microbes for plant disease management has been considered a promising area of research. *Solanum pimpinellifolium*, commonly known as the 'kon-bilahi' in the Assamese language, is a wild species of tomato which have been observed to be tolerant to the bacterial wilt disease which is havoc in the cultivated tomato species i.e. *(Solanum lycopersicum*). The plant is widely grown throughout the Northeastern state of Assam. Numerous sources for resistance to bacterial wilt have been found in *S. pimpinellifolium* since the first discovery of the resistant accession PI127805A (*S. pimpinellifolium*) in 1964 (Acosta *et al.* 1964; Yin *et al.* 2005) [1, 74]. The bacterial wilt of solanaceous crops caused by *Ralstonia solanacearum* is one of the major production constraints of tomatoes and it causes extensive losses in India and Assam.

The yield loss due to bacterial wilt ranged from 11-93 per cent in India (Kishun 1987) [40]. Its occurrence in an epidemic form has been recorded in solanaceous crops from many parts of Assam (Bora, 1995) [14].
We isolated and assessed microbial diversity in rhizospheric soil and plant tissues of tomato plants, and evaluated of its suppressive effect *in vitro* against the bacterial wilt pathogen, *R. solanacearum*. Further, the compatibility of PGPRs and endophytic isolates was tested *in-vitro*. Furthermore, *in vivo* studies were done by applying the combination of beneficial microbes in pot grown tomato plants to obtain a better biological management of bacterial wilt of tomato.

Materials and Methods

Isolation of PGPRs and endophytes

PGPRs were isolated from the rhizosphere of *Solanum lycopericum* and *Solanum pimpinellifolium*. The soil samples were subjected to serial dilution with sterile saline (0.85% w/v NaCl in water) and dilutions were platted on PDA, NA, King's B and Kenknight's Agar and incubated at 28 ± 1 °C for 2-3 days. The CFU appeared were enumerated and the pure cultures of the isolates were preserved in slants as working culture at 4 °C or as glycerol stocks using 50% (v/v) glycerol at -80 °C.
Endophytes were isolated from the healthy aerial parts of the tomato part (Both *Solanum lycopericum* and *Solanum pimpinellifolium)*. The 2-3 cm long sections of the plant tissues were surface sterilized with sodium hypochlorite solution (2-3%) followed by crushing in phosphate buffer using mortar and pestle. The crushed sample was then filtered through a Whattman filter paper no.1. and serial dilutions with sterile saline (0.85% w/v NaCl in water) was done. The dilutions were platted on PDA, NA, King's B and Kenknight's Agar and incubated at 28 ± 1 °C for 2-3 days. The CFU appeared were enumerated and the pure cultures of the isolates were preserved in slants as working culture at 4 °C or as glycerol stocks using 50% (v/v) glycerol at -80 °C.

Characterization of screened isolates

The cultural and morphological characters of the isolates were noted for further studies. The isolates were investigated for their biochemical properties as described in Bergey's Manual of Systematic Bacteriology. For the evaluation of qualitative characters of the PGPR and endophytic isolates, few important tests like production of HCN (Bakker *et al.* 1993) [83], production of ammonia (Lata and Saxena 2003) [84], P solubilisation (Pikovskaya 1948) [78], production of IAA (Bric *et al.* 1991) [79] and production of siderophore (Schwyn and Neilands 1987) [80] was done.

Molecular characterization and phylogenetic analysis

The genomic bacterial DNA was isolated following the modified method of Cardinal *et al.* (1997) [85]. The primer set U16SF-5′-AGAGTTTGATCMTGG CTCAG-3′ and U16SR-5′-TACGGYTACCTTGTTACG ACTT-3′ was used to amplify the 16S rRNA gene. PCR reaction mixture was prepared as per GoTaq® DNA polymer- ase protocol (Promega, USA) with 20 pmols of each primer, 2U Taq DNA polymerase and 100 ng genomic DNA in a final volume of 50 µl and amplification was performed using a thermal cycler (Applied Biosystems, USA). The PCR program was as follows: initial denaturing step of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 1.5 min; a final extension step at 72 °C for 7 min. The amplified products were sequenced by a commercial source (Bioserve Biotechnologies (India) Pvt. Ltd.) The sequence reads of 16S rRNA genes were assembled into contig using CodonCode Aligner (Codon- Code Corporation, USA). Sequence similarity tool BLAST was employed to find the similarity of the sequences with known 16S rDNA sequences in the GenBank database and the sequences were deposited in GenBank of NCBI to attain the accession numbers. Phylogenetic analysis of the isolates was carried out based on 16SrDNA sequences. A phylogenetic tree was constructed using the 16S rDNA sequences of the isolates along with the sequences of other similar strains retrieved from the NCBI GenBank. The sequences were aligned with Clustal W using default parameters and a neighbor joining tree was constructed using MEGA10 software (http://www.megasoftware.net/) with Jukes-Cantor model and 1000-step bootstrap.

Isolation *Ralstonia solanacearum*

Ralstonia solanacearum was isolated from infected tomato plants showing typical disease symptoms from Horticultural Orchard, Assam Agricultural University, Jorhat. It was used to evaluate the efficacy of PGPRs and endophytic isolates *in vitro* and *in plantae.* Pathogenicity test was performed as a part of fulfilling Koch's postulates.

Antimicrobial activity of PGPR and endophytic microbes against *R. solanacearum in vitro*

All the isolated PGPR and endophytic microorganisms were tested *in vitro* for their antagonistic effect against *R. solanacearum* by following dual culture assay of Aspiras and Cruz (1985) [11]. Single colony of freshly grown R. solanacearum grown in nutrient broth for 48 h under agitated condition and the culture was used to seed sterile TTC plates following pour plate technique to obtain uniform bacterial growth. 0.5 cm diameter lawn of the PGPR and/or endophyte isolate grown in NA (For bacteria) or PDA (for fungus) was scooped out with the help of a sterilized cork borer containing cells of the desired isolate and was transferred to the center of TTC plates seeded earlier with *R. solanacearum*. The plates were then incubated at 28±2 °C for 72 hrs. Inhibition zone was measured and percent inhibition calculated. The experiment was conducted with five replications in a completely randomized design (CRD). Streptocycline @200 ppm was also checked for its inhibitory effect against *R. solanacearum* using disc diffusion method (Bauer *et al.* 1966) [13].

Selection and compatibility test

Two PGPR and endophytic isolates each, on the basis of maximum inhibition percentage, were selected. Compatibility among the selected rhizospheric and endophytic isolates was tested *in vitro* by adopting dual culture assay plate technique of Aspiras and Cruz (1985) [11] in culture media taking NA as basal media. The radial growth (mm) of each isolate was recorded individually and in combination upto 72 hrs of incubation. The compatible isolates were selected for further studies to evaluate its effectiveness against the bacterial wilt pathogen *R. solanacearum in vitro*.

In plantae biocontrol assay and statistical analysis

An experiment was conducted using Complete Randomized Design (CRD) to determine the efficacy of selected PGPR and endophytic microbes in controlling bacterial wilt of tomato. For this purpose susceptible variety of tomato (var. Pusa Ruby) was used. Application of the selected PGPR and endophytic microbes as root treatment (100 seedling/100 ml solution), soil treatment (500 ml/plant) and spray application (2% solution), alongside a chemical control check (Streptocycline 0.02%) and an absolute check was performed. The transplanted plants were placed under net house to protect from direct exposure to sunlight. The experiment was carried out in five replications for each treatment and five plants per replication.

All the tomato plants were treated with *R. solanacearum* cell suspension (10^8 cfu/mL) using the root inoculation method described earlier (Singh *et al*. 2018) [63]. The chemical control pots were treated with 0.02% (w/v) Streptocycline and absolute check pots were treated only with the pathogen. The number of wilted plants in each treatment was continuously recorded till 90 days post inoculation with the pathogen. The numbers of completely wilted plants were recorded for each formulation and the percent (%) wilt incidence was calculated.

Results

Isolation PGPR and enophytic microorganisms

In the rhizosphere of *S. pimpinellifolium*, fungal, bacterial and actinomycetes populations varied from (2.73-37.66) x10^4 cfu/g; (9.70- 57.50) x10^7 cfu/g and (6.28- 22.80) x10^5 cfu/g, respectively. While, in the rhizosphere of *S. lycopersicum*, fungal, bacterial and actinomycetes populations varied from (1.86- 9.73) x10^4 cfu/g; (2.33- 23.67) x10^7 cfu/g and (1.73- 11.20) x10^5 cfu/g, respectively.

Table 1: Population of PGPR associated with *S. pimpinellifolium* (kon bilahi) plants and *S. lycopersicum* plants

Sample	*S. pimpinellifolium*			Sample	*S. lycopersicum*		
	Fungi (x10^4 cfu/g)*	Bacteria (x10^7cfu/g)*	Actinomycetes (x10^5 cfu/g)*		Fungi (x10^4 cfu/g)*	Bacteria (x10^7cfu/g)*	Actinomycetes (x10^5 cfu/g)*
RK 1	4.12	25.73	9.72	RT 1	2.65	11.33	9.06
RK 2	16.75	30.27	8.90	RT 2	8.40	23.67	3.76
RK 3	37.66	57.50	22.80	RT 3	4.12	7.33	8.26
RK 4	14.6	13.22	14.47	RT 4	6.53	12.40	11.20
RK 5	2.73	9.70	8.50	RT 5	3.57	8.67	7.33
RK 6	12.26	14.67	10.80	RT 6	1.86	6.72	2.56
RK 7	25.65	41.33	9.62	RT 7	4.33	19.67	1.73
RK 8	6.20	26.70	6.28	RT 8	3.67	2.33	6.40
RK 9	11.50	32.67	18.30	RT 9	9.73	7.20	5.53
RK 10	9.67	11.22	8.07	RT 10	4.67	16.53	3.13

In case of *S. pimpinellifolium* plant, endophytic fungal, bacterial and actinomycetes population varied from (1.70- 11.67) x10^3 cfu/g; (3.57- 14.02) x10^5 cfu/g and (1.35- 11.25) x10^1 cfu/g, respectively. While, in case of *S. lycopersicum* plant, endophytic fungal, bacterial and actinomycetes population varied from (0.83- 5.93) x10^3 cfu/g; (2.16- 7.43) x10^5 cfu/g and (0.23- 6.87) x10^1 cfu/g, respectively.

Table 2: Population of endophytic microorganisms associated with *S. pimpinellifolium* (kon bilahi) plants and *S. lycopersicum* plants

Sample	*S. pimpinellifolium*			Sample	*S. lycopersicum*		
	Fungi (x10^3 cfu/g)*	Bacteria (x10^5 cfu/g)*	Actinomycetes (x10^1 cfu/g)*		Fungi (x10^3 cfu/g)*	Bacteria (x10^5 cfu/g)*	Actinomycetes (x10^1 cfu/g)*
EK1	3.37	5.40	11.25	ET 1	4.68	3.57	3.70
EK2	2.10	14.02	7.45	ET 2	1.46	2.73	1.26
EK3	1.70	6.40	2.37	ET 3	5.93	7.43	6.87
EK4	4.62	7.02	4.72	ET 4	3.10	5.40	0.45
EK5	7.47	3.57	1.35	ET 5	2.23	3.40	3.50
EK6	9.30	7.45	3.95	ET 6	1.46	4.33	1.33
EK7	3.27	6.40	2.67	ET 7	2.56	6.20	0.23
EK8	5.77	13.60	1.52	ET 8	0.83	4.53	1.26
EK9	2.56	5.53	9.37	ET 9	3.30	3.40	2.39
EK10	11.67	10.37	3.27	ET 10	1.75	2.16	4.87

Characterization of the screened isolates

Phenotypic and biochemical characterization

The screened and selected rhizospheric and endophytic isolates were characterized culturally, morphologically and biochemically by colony colour, shape, transparency, pigmentation production, gram reaction of the bacteria and KOH, levan, oxidase, citrate utilization, nitrate reduction, indole production, arginine dihydrolase and starch hydrolysis tests. The PGPR isolate RKB7 was found to be gram positive. It showed negative results to KOH, oxidase, simmon's citrate and levan tests and positive results to starch hydrolysis, nitrate reduction, indole and arginine dihydrolase. The endophytic isolate EKA4 showed gram positive reaction. It was positive to starch hydrolysis, nitrate reduction, indole and arginine dihydrolase tests and negative to KOH, oxidase, simmon's citrate and levan tests. The endophytic isolate EKB6 showed a gram negative reaction. It showed positive results to oxidase, nitrate reduction, indole, simmon's citrate and aginine dihydrolase tests and negative to strach hydrolysis and levan tests.

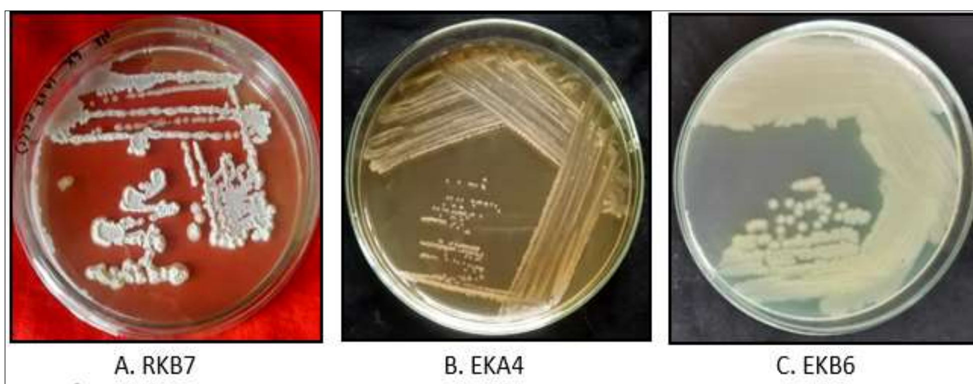

Fig 1: (A-C). Pure culture of the screened rhizospheric and endophytic isolates

The HCN, IAA, ammonia and siderophore production and Zn and P solubilization is related its aggressive antagonistic attribute against plant pathogens. The results in the study revealed that PGPR isolate RKB7 showed positive result for production of IAA (0.215 µg/ml), ammonia, siderophore and solubilization of Zn (2.30 cm). Endophytic isolate EKB6 showed positive result for production of HCN, IAA (0.122 µg/ml), ammonia, siderophore and solubilization of Zn (4.85 cm) and P (2.45 cm) whereas isolate EKA4 showed positive result for the production of production of IAA (0.021 µg/ml), ammonia, siderophore and solubilization of P (1.75 cm) (Table 4.12 and table 4.13).

Table 3: Plant growth promoting metabolites produced by compatible rhizospheric and endophytic isolates

Isolate	HCN production	Ammonia production	Phosphorus production	Zinc production	IAA production	Siderophore production
RKB7	-	+	-	+	+	+
EKA4	-	+	+	+	+	+
EKB6	+	+	+	+	+	+

Table 4: Quantitative data of plant growth promoting metabolities produced by compatible rhizospheric and endophytic isolates

Isolate	Phosphorus production	Zinc production	IAA production
	Diameter of halo zone (cm)	Diameter of halo zone (cm)	IAA (mg/l)
RKB7	negligible	2.30	0.215
EKA4	1.75	negligible	0.021
EKB6	2.45	4.85	0.122

Molecular characterization and phylogenetic analysis

To identify the bacterial isolates, 16S rRNA sequencing was performed. Phylogenetic analysis based on the 16S rRNA sequences of the isolates and their closest matches from NCBI showed highest homology with *Bacillus* species for RKB7 isolate, *Streptomyces* species for EKA4 and *Pseudomonas* species for EKB6 isolate as revealed by the grouping patterns in the maximum likelihood method. The analysis of 16S rDNA gene sequences and morphological, cultural, biochemical studies revealed the RKB7 isolate as *Bacillus subtilis* (ON261568.1), EKA4 as *Streptomyces virginiae* (ON223266.1) and EKB6 as *Pseudomonas aeruginosa* (ON248938.1).

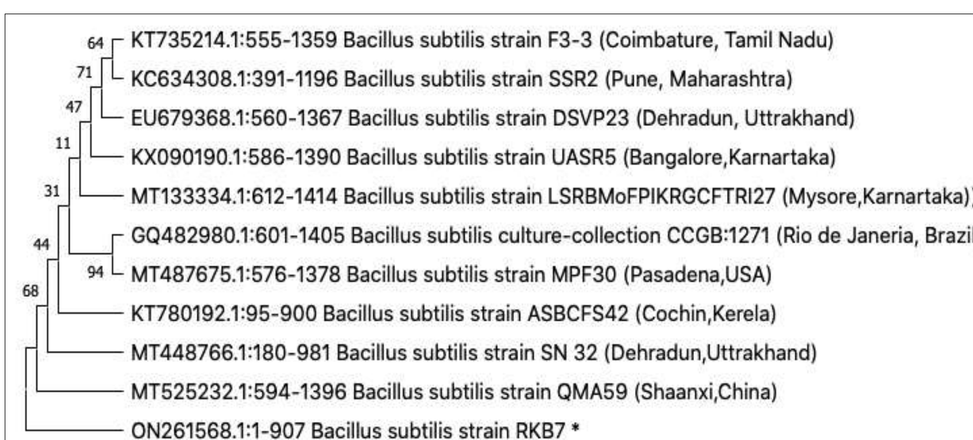

Fig 2: Neighbour-joining phylogenetic tree prepared from the 16S rDNA sequences of RKB7 isolates along with the 16S rDNA sequences of other related species from NCBI using MEGA 10.

Fig 3: Neighbour-joining phylogenetic tree prepared from the 16S rDNA sequences of EKA4 isolates along with the 16S rDNA sequences of other related species from NCBI using MEGA 10.

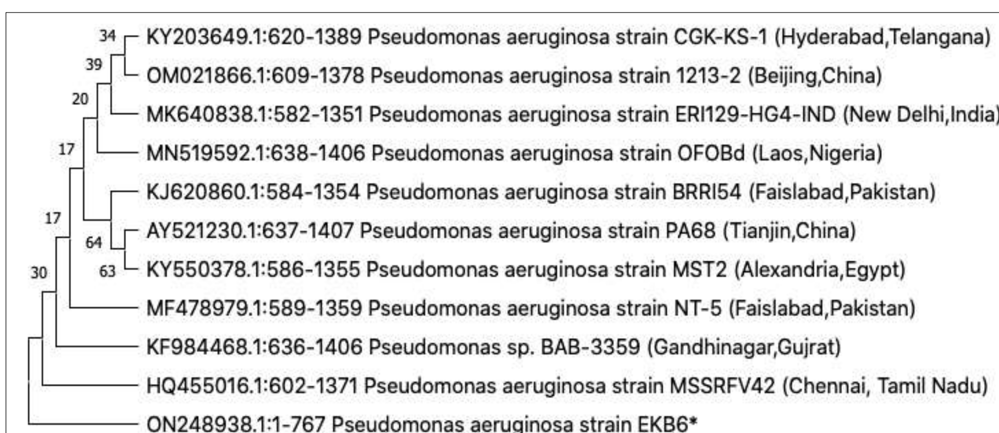

Fig 4: Neighbour-joining phylogenetic tree prepared from the 16S rDNA sequences of EKB6 isolates along with the 16S rDNA sequences of other related species from NCBI using MEGA 10.

Antagonism of PGPR and endophytic isolates against *R. solanacearum*

The antagonist potential of isolated PGPR and endophytic isolates was tested against *R. solanacearum* using modified dual culture assay. The highest inhibition of PGPR was seen in RKB7 isolate (52.96 mm) with 58.83% growth suppression. Similarly, in case of endophytes, the highest inhibition was shown by the isolate EKB6 (36.97 mm) with 41.08% growth suppression (Table 5).

Table 5: Suppressive effect of PGPR and endophytic isolates against *R. solanacearum*

Isolation type	PGPR isolate	Radial growth (mm)	Inhibition percentage (%)	Endophytic isolate	Radial growth (mm)	Inhibition percentage (%)
S. pimpinellifolium (bacteria)	RKB2	26.6	29.85 (33.09)*	EKB2	18.67	20.74 (27.06)*
	RKB7	52.95	58.83 (50.07)	EKB6	36.97	41.08 (39.87)
	RKB10	32.1	35.67 (36.69)	EKB7	15.4	17.11 (24.43)
				EKB10	12.16	13.51 (21.56)
S. pimpinellifolium (actinomycete)	RKA5	17.4	19.33 (26.06)	EKA4	30.13	33.47 (35.37)
	RKA7	19.5	21.67 (27.76)	EKA5	19.5	21.67 (27.76)
S. pimpinellifolium (fungi)	RKF1	14.23	15.81 (23.42)	EKF2	18.53	20.60 (26.99)
	RKF3	19.16	21.29 (27.49)			
S. lycopersicum (bacteria)	RTB4	15.56	17.29 (24.58)	ETB1	23.9	26.56 (31.05)
	RTB6	19.7	21.89 (27.90)	ETB2	11.54	12.82 (20.96)
S. lycopersicum (actinomycete)	RTA3	10.67	11.85 (20.09)	ETA3	17.1	19 (14.70)
S. lycopersicum (fungi)				ETF1	24.83	27.59 (31.69)
	S.Ed (±)		1.14	S.Ed (±)		1.26
	C.D$_{0.05}$		2.41	C.D$_{0.05}$		2.68

*Data in parenthesis represents angular transformation

On the basis of highest antagonism observed, two PGPR isolates (RKB7 and RKB10) and two endophytic isolates (EKA4 and EKB6) were tested for their mutual compatibility. Three isolates *viz.* RKB7, EKA4 and EKB6 were found compatible with each other while RKB10 isolate was found to be incompatible with all the other isolates. The antagonistic potential of RKB7, EKA4 and EKB6 isolates and their combinations were tested against *R. solanacearum* adopting dual culture method using TTC as basal medium. Streptocycline @200 ppm was also checked for its inhibitory effect against *R. solanacearum* using disc diffusion method. *R. solanacearum* cultured in TTC medium served as control. The inhibition produced by the combination of three isolates RKB7, EKA4 and EKB6 and was significantly highest (72.14%) followed by combination of two isolates RKB7 and EKB6 (62.42%). The lowest inhibition was shown by the isolate EKA4 alone (30.13%). Streptocycline @ 200 ppm was also performed as an antibiotic check, which produced an inhibition of 31.85% (Table 8).

Table 6: Suppressive effect of the compatible rhizospheric and endophytic isolates and their combinaton against *R. solanacearum in-vitro*

Treatment No.	Treatment	Inhibition zone (mm)	Inhibition percentage (%)
T_0	*R. solanacearum* (control)	0.00	0.00 (1.04)*
T_1	RKB7	52.95	58.83 (50.07)
T_2	EKA4	27.12	30.13 (33.27)
T_3	EKB6	36.97	41.08 (39.87)
T_4	RKB7+EKA4+EKB6	64.92	72.14 (58.12)
T_5	RKB7+EKA4	52.50	58.33 (49.78)
T_6	RKB7+EKB6	56.18	62.42 (52.18)
T_7	EKA4+EKB6	44.45	49.38 (44.66)
T_8	Streptocycline (200 ppm)	28.67	31.85 (34.33)
	S.Ed (±)		0.49
	$C.D_{0.05}$		1.03

*Data in parenthesis represents angular transformation

In plantae biocontrol assay of _R. solanacearum_ using screened isolates and their combination

The effect of application of the PGPR and endophytic isolates on wilt incidence in tomato, revealed that, the disease incidence decreased significantly accompanied by significant increase in yield of the plants. The PGPR and endophytic isolates and their combination were applied as combinations of root, soil and spray treatment, alongside a chemical control check (Streptocycline 0.02% (w/v)) and an absolute check. The lowest disease incidence was exhibited by the combination of RKB7, EKA4 and EKB6 (31.33%) applied as root treatment, soil application and foliar spray followed by the treatment with combination of RKB7 and EKB6 (35.56%), streptocycline @ 200 ppm (40%), RKB7 and EKA4 (47.78%), EKA4 and EKB6 (55.56%), RKB7 (62.22%) and EKB6 (71.11%), respectively. Highest yield was recorded in plants treated with combination of RKB7, EKA4 and EKB6 (340.67 g/plant) followed by the treatment with combination of RKB7 and EKB6 (305.33 g/plant), streptocycline @ 200 ppm (278.67 g/plant), RKB7 and EKA4 (248.33 g/plant), EKA4 and EKB6 (226.67 g/plant), RKB7 (218.33 g/plant) and EKB6 (189.67 g/plant), respectively. In contrast, the untreated plants showed the highest disease incidence (80%) and lowest yield (93.33 g/plant) (Table 9).

Table 7: Efficacy of rhizospheric and endophytic isolates and their combination on bacterial wilt incidence and yield of tomato

Treatment	Disease incidence (%)	Disease reduction (%)	Yield (g/plant)	Yield increase over control
T_1 = RKB7	62.22 (52.06)*	22.23 (28.11)	218.33	133.93
T_2 = EKB6	71.11 (57.48)	11.11 (19.46)	189.67	103.22
T_3 = RKB7+EKA4+EKB6	31.11 (33.90)	61.11 (51.14)	340.67	265.01
T_4 = RKB7+EKA4	47.78 (43.74)	40.28 (39.35)	248.33	166.07
T_5 = RKB7+EKB6	35.56 (36.63)	55.55 (48.16)	305.33	227.15
T_6 = EKA4+EKB6	55.56 (48.22)	30.55 (33.52)	226.67	142.86
T_7 = Streptocycline @ 200 ppm	40.00 (39.23)	50.00 (45.00)	278.67	198.58
T_8 = control	80.00 (63.43)	-	93.33	-
S.Ed (±)	0.56		7.78	
$C.D_{(P=0.05)}$	1.20		16.25	

*Data in parenthesis represents angular transformation

The yield attributing characters like plant height, No. of leaves, No. of primary branches, No. of fruits per plant, root length, root and shoot dry weight of tomato plants were recorded and it was revealed that they increased significantly in different treatments comprising of combination of bioagents (Table 10). The best treatment was found to be that of RKB7+EKA4+EKB6 followed by RKB7+EKB6, RKB7+EKA4, EKA4+EKB6, RKB7, EKB6, respectively.

Table 8: Efficacy of rhizospheric, endophytic isolates and their combination on yield attributing characters of tomato plant

Treatments	Yield attributing characters							
	Plant height (cm)	Root length (cm)	No. of leaves	No. of primary branches	Shoot dry weight (g)	Root dry weight (g)	Root fresh weight (g)	No. of fruits per plant
T_1 = RKB7	57.40	18.67	72.16	7.50	20.50	1.73	7.50	5.26
T_2 = EKB6	55.53	16.43	63.83	7.13	17.50	1.67	7.30	4.63
T_3 = RKB7+EKA4+EKB6	74.16	27.50	92.5	10.13	30.40	3.16	10.76	7.06
T_4 = RKB7+EKA4	62.50	21.67	81.83	8.30	25.10	2.00	8.67	6.10
T_5 = RKB7+EKB6	66.67	25.50	88.33	8.67	23.53	2.77	9.87	6.73
T_6 = EKA4+EKB6	59.83	19.50	77.00	7.86	26.20	1.75	7.56	5.67
T_7 = Streptocycline @ 200 ppm	65.23	23.33	86.00	8.16	22.80	2.14	9.10	6.30
T_8 = control	43.83	13.10	42.33	6.34	11.83	1.40	6.73	2.20
S. Ed (±)	1.53	0.78	1.52	0.34	0.76	0.36	1.09	0.36
$CD_{0.05}$	3.25	1.65	3.22	0.72	1.62	0.77	2.32	0.78

Discussion

The biological control using plant beneficial microbes *viz*., PGPRs and endophytes against plant pathogens has a great potential as alternative for chemical pesticides for sustainable agriculture. In this study, PGPRs and endophytes from wild tomato species *Solanum pimpinellifolium* and commercially cultivated tomato species *Solanum lycopersicum* were isolated and screened based on their antimicrobial activity against bacterial wilt pathogen *Ralstonia solanacearum*. One PGPR (RKB7) and two endophytes (EKB6 and EKA4) showed the mutual compatibility and greatest suppression against *R. solanacearum* amongst all. Based on the 16SrRNA sequence analysis, RKB7 showed maximum homology with *Bacillus subtilis* (accession number- ON261568.1), EKA4 with *Streptomyces virginiae* (accession number- ON223266.1) and EKB6 as *Pseudomonas aeruginosa* (accession number- ON248938.1).

Bacillus is one of the most potential biocontrol genera due to its varied mechanisms such as the ability to form spores (Liu and Sinclair 1993) [47], produce antimicrobial metabolites (van Loon 2007) [65], formation of biofilm (Kinsinger *et al.,* 2003) [61] and induce host systemic resistance (Choudhary and Johri, 2009) [83]. The mechanisms of the antagonism of *Pseudomonas* seem to be competition with pathogenic microorganisms for iron by the release of siderophores which are secondary metabolites with a strong affinity of $Fe3+$ (Kloepper *et al.* 1980) [41], production of a wide range of metabolites like antibiotics (Fravel 1988) [23], siderophores (Loper and Buyer 1991) [48] and other substances such as cyanide (Voisard *et al.* 1989) [66]. *Streptomyces* spp. are active producers of antibiotics and volatile organic compounds, both in soil and *in planta*. Biocontrol activity shown by the non-pathogenic strain of *Streptomyces* spp. might be due to antibiosis or competition for space or nutrients in the rhizosphere (Whipps 2001) [69]. The suppressive effect of RKB7, EKB6 and EKA4 shown in our study against *R. solanacearum* might be due to similar mechanisms and production of similar types of metabolites.

The quantitative estimation of rhizospheric and endophytic microorganisms in the *Solanum pimpinellifolium* plant revealed that bacteria dominated both spheres, outnumbering fungi and actinomycetes. In a study conducted by Chinakwe *et al. (*2019) [19] recorded the rhizospheric bacterial count of 7×107 to 4.5 ×109 cfu/g and fungal count of 1.2×106 to 8.7 ×106 cfu/g for cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) and 5.4×107 to 3.0 ×109 cfu/g and fungal count of 1.0×106 to 1.2 ×106 cfu/g for plum tomato (*Solanum lycopersicum*). Singh *et al. (*2018) [63] evaluated endophytic bacterial population of tomato plants in 6 hybrid varieties (Kashi Sharad, Kashi Anupam, Kashi Amrit, DVRT-2, Prestige and S- 22) and 2 local varieties (S-3619 and Kajla). Results revealed that bacterial population varied from 2.8x103 -3.5x105 cfu/g. The highest bacterial population (3.5x105 cfu/g) was recorded for local variety S-3619 and the lowest (2.8x103 cfu/g) for hybrid variety Kashi Sharad.

The results of compatibility tests revealed that most of the isolates were compatible with each other except the isolate RKB10. The possibility of incompatibility of rhizospheric and endophytic isolates has been reported by many researchers. James and Mathew (2007) [36] reported that only three out of five endophytic bacterial isolates isolated from the tomato plants were mutually compatible. Albareda *et al.* (2006) reported mutual incompatibility between rhizobacterial strains *Pseudomonas fluorescens* and *Chryseobacterium balustinum.* Such studies reveal that microbial antagonists might or might not be mutually compatible with each other.

The cultural, morphological and biochemical tests performed for the PGPR and endophytic isolates in our study, the results of which were in confirmatory to studies conducted by various researchers. The PGPR isolate RKB7 (*Bacillus subtilis*) showed positive responses in production of IAA, ammonia, siderophore and solubilization of Zn. Wani and Khan (2010) [68] reported similar results of IAA and ammonia for *Bacillus* species. Zaidi *et al.* (2006) [75] recorded positive results for IAA production and P solubilization in *Bacillus subtilis* strain SJ 101 isolated from Indian mustard plant, *Brassica juncea*. Endophytic EKB6 isolate (*Pseudomonas aeruginosa)* showed positive response in the production of HCN, IAA, ammonia, siderophore and solubilization of Zn (4.85cm) and P (2.45cm). Noori and Saud (2012) [57] isolated 20 strains of *Pseudomonas fluorescens* and found similar results for the production of IAA, siderophores and HCN. Out of bacterial isolates, ammonia production was detected in 92% of isolates of *Pseudomonas* (Sakthivel and Karthikeyan, 2012) [62]. Endophytic isolate EKA4 (*Streptomyces viginiae)* showed positive response for the production of IAA, ammonia, siderophore and solubilization of P. Anwar *et al.* (2016) [10] isolated 98 strains of actinomycetes from wheat and tomato rhizosphere and reported 6 Streptomyces strains to be potential IAA, HCN and ammonia producers. Out of 320 rhizobacterial isolates, maximum phosphate solubilizing activity was shown by 3 *Streptomyces* isolates *viz*., *Streptomyces* isolate L3, *Streptomyces* isolate KT 6-4-1 and *Streptomyces* isolate ST 3 (Chaiharn *et al.* 2018). The antagonistic potential of PGPR (*Bacillus subtilis*) and endophytic isolates (*Pseudomonas aeruginosa* and *Streptomyces virginiae*) was tested against bacterial wilt pathogen of tomato, *R. solanacearum*. The effectiveness of

these microbes in suppressing *R. solanacearum* has been demonstrated by various researchers. (Tan *et al.,* 2006; Ramesh and Phadke, 2012; Yendyo *et al.,* 2017) [64, 61, 73].

In plantae study revealed that the lowest disease incidence 31.11% and the highest fruit yield (340.67 g/plant) was observed when microbes are applied in combination (RKB7+EKB6+EKA4). Alongside, this combination also showed the best yield attributing characters like plant height, plant height, No. of leaves, No. of primary branches, No. of fruits per plant, root length, root and shoot dry weight. Combinations of endophytes and plant growth promoting rhizobacteria (PGPR) can enhance disease suppression (Guetsky *et al.* 2002) [25], improve crop yields and enhance nutrient uptake by plants (Alagawadi and Gaur 1992) [4] over single organism inoculations. Ramesh *et al,* 2009 [61] recorded (25-63)% bacterial wilt incidence when different endophytic isolates of *Pseudomonas* spp. were applied as seed treatment and seedling root dip treatment on bacterial wilt infected eggplant. James (2015) recorded 26.19% wilt incidence on application of endophytic microbial consortium (*Trichoderma harzianum, T. viridi*-1, *T. viridi*-2, *Bacillus subtilis, Streptomyces thermodiastaticus*) applied as seed treatment+ seedling root dip + soil drenching under *in-planta* condition against bacterial wilt of tomato. Plant-growth-promoting Pseudomonas strains, according to Van Peer and Schippers (1989) [86], increase root and shoot mass in tomato, cucumber, lettuce, and potato, most likely by suppressing detrimental rhizosphere microflora. Hashem *et al.* 2019 [31] documented that the indirect biocontrol mechanism of *Bacillus subtilis* comprises plant growth and induction of acquired systemic resistance. Amini *et al.* 2016 [7] reported that the biomass of chickpea plants (Plant height and dry weight) significantly increased in plants treated with *Streptomyces* strains compared to non- bacterized control.

Conclusion

Characterization and 16SrDNA sequence analysis of the PGPR and endophytic isolates helped to screen out the potential biocontrol microbes for the management of bacterial wilt of tomato. The study showed that the isolates had the capacity to produce HCN, IAA, ammonia, siderophore and solubilize Zn and P which resulted in plant growth promotion and antagonism. Also, PGPR and endophytic isolates from local wild species of tomato, *Solanum pimpinellifolium* showed greater antagonism in comparison to those isolated from *Solanum lycopersicum* and thereby being promising candidates as biocontrol agents for management of bacterial wilt disease caused by *Ralstonia solanacearum*. Such wild relatives of plant species growing naturally in our environment are of much importance and these must be preserved and utilized to seek solutions to problems such as plant diseases. Although, further analysis to identify the compounds responsible and their mode of action are necessary.

Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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