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INTEGRATION OF RHIZOBACTERIAL ISOLATES AND AIRONE CHEMICAL FOR EFFECTIVE MANAGEMENT OF BACTERIAL WILT IN CUCUMBER (*CUCUMIS SATIVUS*)

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ABSTRACT

The present study aimed to evaluate the efficacy of integrated disease management strategies against bacterial wilt, caused by *Erwinia tracheiphila* in cucumber (*Cucumis sativus*) within a controlled greenhouse environment. A total of 56 *E. tracheiphila* were recovered from the symptomatic cucumber plants among which 13 were tested highly virulent. Among six rhizobacterial isolates; *Pseudomonas flurescens*-3 (Pf-3), *Pseudomonas putida*-5 (Pu-5), *Pseudomonas stutzeri*-2 (Ps-2), *Bacillus subtilis*-1 (Bs-1), *Bacillus safensis*-2 (Bs-2), *and Pseudomonas stutzeri*-1 (Ps-1), tested *in vitro* using dual culture technique against extremely virulent strain of *E. tracheiphila* revealed Pf-3, Pu-5 and Bs-1 significantly reduced its growth. Two separate experiments were performed to investigate the synergistic effects of these PGPRs in combination with Airone chemical (active ingredients; Copper Oxychloride + Copper Hydroxide 20%SC by Swat Agro Chemicals, Pakistan) on disease severity and overall plant growth. In the first experiment, eight treatments were tested in a complete randomized design (CRD) with eight replications, focusing on the combination of Pf-3, Pu-5 and Bs-1. Results revealed that the combined application of Pf-3 and Pu-5 significantly outperformed other treatments, exhibiting substantial improvements in key growth parameters; vine length, number of leaves and branches per plant, and a remarkable reduction in disease severity compared to positive and negative controls. In the second experiment, Pf-3, Pu-5 and Bs-1 and Airone chemical were employed in seed and soil treatments to confer resistance to *E. tracheiphila* and suppress bacterial wilt. The treatment involving P.u-5 and Bs-1, along with a foliar spray of Airone, recorded the lowest disease severity and an increase in plant growth compared to the positive control. These findings suggest that the synergistic application of PGPR and Airone chemical holds promise for integrated disease management in cucumber, providing effective control of bacterial wilt while promoting plant growth. Moreover, the environmentally friendly nature of rhizobacterial-based formulations underscores their potential as safe alternatives for controlling soil-borne plant pathogens without adverse effects on human health or the environment.

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INTRODUCTION

The bacterial wilt of cucumber is a significant disease affecting cucumber crops, posing a growing concern for producers in Pakistan. This disease is caused by the

bacterial pathogen *Erwinia tracheiphila* (Shapiro *et al.*, 2018). Cucumber (*Cucumis sativus* L.) belongs to the crucial Cucurbitaceae family, and both cucumber and muskmelon (*Cucumis melo*) within this family are susceptible to this deadly disease. Bacterial wilt of cucumber has become a prominent vegetable disease in Pakistan, previously overlooked and lacking special attention. The transmission of the pathogen *E. tracheiphila* occurs through cucumber beetles, specifically the striped cucumber beetle (*Acalymma vittatum* F.) and spotted cucumber beetles (*Diabrotica undecimpunctata*) (Shapiro *et al.*, 2018).

Studies indicate that approximately 7-10% of overwintering cucumber beetles test positive for *E. tracheiphila* (Fleischer *et al.*, 1999). The disease cycle initiates with these infected cucumber beetles. Cucumber beetles not carrying the pathogenic bacterium can become carriers after feeding on infected plants. The pathogenic bacteria overwinter in the foregut and hindgut of cucumber beetles, multiplying as needed (Garcia-Salazar *et al.*, 2000). Infested cucumber beetles, when feeding on a healthy plant, introduce the pathogenic bacteria into the plant through wounds from their excrement (Sasu *et al.*, 2010). Once inside a healthy cucumber plant, *E. tracheiphila* multiplies in the xylem, leading to the blockage of water and nutrient supply and subsequent wilting of the plant (Rocha *et al.*, 2019).

In an effort to combat this disease, some progressive farmers in Pakistan refrain from using chemicals on their crops. They employ a method of covering cucumber plants with polythene sheets immediately after transplantation, removing the cover when flowers appear to allow for pollination. However, this method proves ineffective, as cucumber beetles in the field attack the plants once the cover is removed.

E. tracheiphila and cucumber beetles can thrive on various plants (Bassi Jr, 1982). The pathogen can endure in the mid-gut of a cucumber beetle for several years during dormancy (Mohammadi and Sharifi, 2016). When temperatures are favorable, beetles emerge from the soil and are drawn to cucumber plants (Elsey, 1989). Cucurbits, especially cucumber and muskmelon, produce chemical compounds like cucurbitacin that influence the feeding behavior of cucumber beetles (Lewis *et al.*, 1990).

Bacterial wilt disease is challenging to control but can be mitigated through cultural practices such as late cultivation, clearing surplus vegetation near the field, and thorough plowing before plant transplantation (Atiq *et al.*, 2022). However, the threat remains, as *E. tracheiphila* and cucumber beetles can destroy crops when exposed to suitable temperatures for an extended period (Rojas *et al.*, 2015).

Chemical management can help manage cucumber bacterial wilt, especially when disease incidence is high and cultural control is insufficient (Raupach and Kloepper, 1998). However, these control strategies can harm the environment and humans. Chemical control measures for cucumber bacterial wilt can cause residue buildup in the soil and crops, which can harm soil health, crop productivity, and human health. Chemical pesticides can pollute the soil, air, and water and harm non-target organisms, and frequent and indiscriminate use can increase pesticide-resistant pathogens and vectors, which can reduce the efficacy of chemical control measures (Pecenka *et al.*, 2021). Thus, it is crucial to carefully evaluate chemical control measures for bacterial diseases and adopt an integrated approach that includes cultural and chemical control measures, as well as good agricultural practices that minimise their negative effects.

Biological control, particularly using plant growth-promoting rhizobacteria (PGPR) in the rhizosphere, proves essential in disease management. This approach not only helps reduce the reliance on harmful chemicals but also aligns with environmentally friendly strategies. Our research aims to explore the protective potential of PGPRs alone and in combination with Airone, a most frequent use chemical against bacterial wilt of cucumber, offering an eco-friendly alternative for safeguarding cucumber crops.

MATERIALS AND METHODS

Pathogen Isolation

Infected leaves and vine segments of cucumber plants showing symptoms of bacterial wilt, such as wilting and yellowing of leaves (Yao *et al.*, 1996), were pruned with pre-sterilized scissors and blades. The collected samples were placed in clean, sealable plastic bags or containers and labelled with the date, location, and plant variety. These infected plant portions were surface sterilized using 1% NaOCl, followed by three consecutive rinses with distilled water and damp dried on paper towels. Subsequently, the treated segments were placed on solidified nutrient agar (NA) culture media in Petri dishes incubated at 28 ± 2°C for 02-03 days, following

established protocols (De Mackiewicz *et al.*, 1998; Mitchell and Hanks, 2009).

The meticulous selection of colonies resembling *E. tracheiphila* in morphology were transferred to new NA containing Petri plates incubated at the optimal temperature. The purified bacterial colonies were then preserved in a 50% glycerol solution at -20°C for future experimentation (Rojas and Gleason, 2012). The pathogenic bacteria were identified using a comprehensive approach that included the examination of physiological properties using microscopy techniques and comparison with previously documented bacterial strains in the literature (De Mackiewicz *et al.*, 1998).

Preparation of Bacterial Inoculum

A single colony of *E. tracheiphila* was selected, inoculated into sterile nutrient broth (NB) medium incubated at 28± 2°C for 18-24 hours with constant shaking at 150-200 rpm until the culture reached the logarithmic growth phase. The bacterial culture was then diluted with sterile distilled water to achieve the desired inoculum concentration (1×10^8 CFU/ml) (Stockwell *et al.*, 1998). To verify the bacterial concentration of the inoculum, sample was taken, and serial dilutions were made in sterile distilled water. These diluted samples were plated on nutrient agar plates and incubated at 28± 2°C for 24-48 hours. Colony counts on the plates determined the bacterial concentration (Kaur *et al.*, 2019).

Pathogenicity

Seeds of the commonly cultivated varieties of cucumber (*Cucumis sativus*), i.e., Kheera local, Punjab Kheera, Yousaf F1, and Adventa 703, were grown in pots. At four weeks, inoculum of *E. tracheiphila* was prepared in sterile nutritional broth. Leaves and stems of the healthy plants were surface sterilized with 70% ethanol and inoculation was done by injecting 1 cc of inoculum into plant leaves or stems (Klement and Goodman, 1967). The control treatments used simply distilled water. Plants were observed for wilting, yellowing, and tissue necrosis (Liu *et al.*, 2018). *E. tracheiphila* was reisolated from the disease plant portions on NA medium. Biochemical tests and molecular identifications were made to confirm the identity of the pathogen.

Biochemical Analysis

The identification of highly virulent *E. tracheiphila* isolates using biochemical characterization involved following the methodology outlined by Khasabulli *et al.* (2017). A series of biochemical tests were performed including gram staining, oxidase, Catalase, Citrate Utilization, Methyl red, Indole, Voges-Proskauer, Urease and Nitrate Reduction test.

Molecular Analysis

Genomic DNA extraction from 13 highly virulent isolates of *E. tracheiphila* was carried out using the GeneJet DNA Extraction Kit (Thermo Scientific) following the manufacturer's standard protocol (Malapelle *et al.*, 2022). Amplification 16 rRNA was done using a set of forward and reverse primers; 1F [5′AAACTCTAAAGTGAATTGACGG3′] and 1R [5′ACGGGCGGGTGTGTAGC 3′]. The resulting PCR product underwent electrophoresis on a 1% agarose gel, and the expected band was subsequently purified using the Genejet PCR Purification Kit protocol (Thermo Scientific).

Sequences obtained from MacroGen Korea were further processed using the BioEdit program. The sequence alignments were conducted using the Clustal W program (Thompson *et al.*, 1997). Further, the aligned sequences were cross-referenced for similarity with previously submitted gene sequences using BLAST on the NCBI database to confirm the species. Phylogenetic analysis was performed using the maximum likelihood method with 1000 bootstrap replicates on the software, MEGA version 7.0 (Tamura *et al.*, 2013).

Selection of Rhizobacterial Isolates

Six previously reported rhizobacterial isolates; *Pseudomonas flurescens*-3 (Pf-3), *Pseudomonas putida*-5 (Pu-5), *Pseudomonas stutzeri*-2 (Ps-2), *Bacillus subtilis*-1 (Bs-1), *Bacillus safensis*-2 (Bs-2), and *Pseudomonas stutzeri*-1 (Ps-1) were obtained from the Department of Plant Pathology at PMAS-Arid Agriculture University Rawalpindi, Pakistan. These isolates and seven highly virulent isolates of *E. tracheiphila* were streaked onto separate nutrient agar plates incubated at 28 ºC for 24-48 hours. The growth of each isolate was observed and assessed for purity. Subsequently, a fresh inoculum was prepared in nutrient broth medium by incubating the isolates separately. Nutrient broth containing *E. tracheiphila* was spread on each agar plate, and a disc of sterilized filter paper dipped in nutrient broth containing rhizobacteria was placed in the middle of each plate. After incubating for 24-48 hours at 28 ºC, the plates were examined for evidence of inhibition of *E. tracheiphila* growth by each rhizobacterial isolate. The isolates showing the highest degree of inhibition were chosen for further study.

Rhizobacterial Compatibility Test

Rhizobacterial isolates were inoculated separately in sterilized culture tubes containing nutrient broth incubated at 28°C. Solidified nutrient agar plates were prepared for testing compatibility among selected rhizobacterial isolates. With a sterile cotton swab, bacterial strains were separately scattered across each plate. 6 mm sterile filter paper disks soaked with other rhizobacteria containing nutrient broth solution and place in the middle of the plates incubated for 48 hours at 28°C. After incubation, the plates showed growth inhibition zones or interactions between the each, two bacterial strains. When both bacterial strains grew uniformly on the plate without inhibitory zones, they were considered compatible. A growth inhibition zone around the wet filter paper disk revealed the strains were incompatible.

Management of *Erwinia tracheiphila* Infection

Two distinct experiments were performed under controlled greenhouse conditions to assess the effectiveness of selected rhizobacterial strains in combating *Erwinia tracheiphila* infection in cucumber. The first experiment, known Biological Control, was conducted by incorporating 8 different treatments with 8 replications for each treatment. In this experiment, plant growth-promoting rhizobacteria were employed to trigger systemic resistance in cucumber plants against the bacterial pathogen responsible for cucumber bacterial wilt. Three compatible and highly effective rhizobacteria were applied both individually and in combination to combat *E. tracheiphila*. The second experiment, named as Integrated Disease Management, involved the combined application of plant growth-promoting rhizobacteria and Airone chemical. This approach aimed to assess the combined impact of these treatments on the disease parameters associated with bacterial wilt disease in cucumber.

Biological Control

Three highly effective and compatible rhizobacteria; *Pseudomonas flurescens* (Pf-3), *Pseudomonas putida* (Pu-5), and *Bacillus subtilis* (Bs-1) were applied individually and in combination to suppress *E. tracheiphila* infection in cucumber. 4 weeks old growing seedlings of a highly susceptible cucumber variety having 4-5 true leaves were transplanted into pots containing autoclaved soil and peat moss. Plants were kept in a greenhouse where they were irrigated every other day and received weekly doses of liquid fertilizer with a composition of N:P:K 15:15:15. The day and night temperature in greenhouse was maintained as 30°C to 35°C and 23°C to 28°C respectively.

The inoculum of the most virulent strain of *Erwinia tracheiphila* was maintained on solid nutrient agar pepton (NAP) medium. Colonies of *E. tracheiphila* were harvested from the surface of solidified medium and suspend in 10 mM phosphate-buffered saline (PBS) to prepare bacterial suspension at a concentration of approximately 1 x 10^8 CFU/ml. Similarly, the rhizobacterial strains suspensions was maintained at 1 x 10^8 CFU/ml by diluting with distilled water.

After transplanting seedlings to pots, 5 ml of each PGPR solution containing 1 x 10^8 CFU/ml was drenched into the roots. One week following the initial dosage, the plants received a second injection of PGPR at the same concentration. Two weeks after transplanting seedlings in pots, 1 ml pathogenic bacterial inoculum solution containing 1 x 10^8 CFU/ml was injected using syringe into each plant stem. Plants were visited weekly to record data on disease development and plant growth parameters.

The treatments used were as follows:

- T1-Negative control , no pathogenic bacteria and no PGPR applied abbreviated as - CON
- T2-Positive control , only pathogenic bacteria applied abbreviated as + CON
- T3-Pathogenic bacteria and *Pseudomonas flurescens* applied abbreviated as PBR1
- T4-Pathogenic bacteria and *Pseudomonas putida* applied abbreviated as PBR2
- T5-Pathogenic bacteria and *Bacillus subtilis* applied abbreviated as PBR3
- T6-Pathogenic bacteria and combination of *P. flurescens* & *P. putida* applied abbreviated as PBR1-2
- T7-Pathogenic bacteria and combination of *P. flurescens* & *B. subtilis* applied abbreviated as PBR1-3
- T8-Pathogenic bacteria and combination of *P. putida* & *B. subtilis* applied abbreviated as PBR2-3.

Integrated Disease Management

The experiment was conducted in a greenhouse under the same controlled conditions, using three compatible rhizobacterial individually, double PGPR combinations and a commercial insecticide, Airone, containing copper oxychloride and copper hydrochloride. Cucumber plants, treated with PGPRs, were grown in seedling trays having autoclaved soil and peat moss.

Plants received irrigation every other day weekly dose of liquid fertilizer with a composition of N:P:K 15:15:15. After two weeks, cucumber plants were inoculated with

a pathogenic bacterial suspension, followed by two applications of Airone, seven days apart. The recommended concentration of 160 ml per acre was used for foliar application using a hand-held sprayer until runoff, aiming to observe the combined impact of Airone and PGPR on cucumber bacterial wilt disease.

The treatments used were as follows:

- T1-Negative control, no pathogenic bacteria, no PGPR and no chemical applied abbreviated as – CON
- T2-Positive control, only pathogenic bacteria applied abbreviated as + CON
- T3-Pathogenic bacteria, *Pseudomonas flurescens* and airone applied abbreviated as PBR1 + Airone
- T4-Pathogenic bacteria, *Pseudomonas putida* and airone applied abbreviated as PBR2 + Airone
- T5-Pathogenic bacteria, *Bacillus subtilis* and airone applied abbreviated as PBR3 + Airone
- T6-Pathogenic bacteria, combination of *P. flurescens* & *P. putida* and Airone applied abbreviated as PBR1-2 + Airone
- T7-Pathogenic bacteria, combination of *P. flurescens* & *B. subtilis* and Airone applied abbreviated as PBR1-3 + Airone
- T8-Pathogenic bacteria, combination of *P. putida* & *B. subtilis* and Airone applied abbreviated as PBR2-3 + Airone

Statistical analysis

A Completely Randomized Design (CRD) was employed for the experiment, involving eight treatments with eight replications each. The data collected from the management trials were subjected to analysis using the ANOVA test. Post-analysis, Tukey's Honestly Significant Difference test (HSD) was utilized to compare the means of the treatments.

RESULTS

Pathogen (*Erwinia tracheiphila*)

Symptoms of bacterial wilt appeared as drooping or witling of one or few vine leaves. Later the entire vine wilted and dried up. In less vulnerable plants symptoms emerged slowly (Komm and Agrios, 1978). When stems were pressed between fingers until the formation of white ooze. The fragile threads stretching to several centimeters confirmed the characteristic symptoms of bacterial wilt (Sherf and MacNab, 1986). The pathogen was isolated from the symptomatic plant portions on NA medium. A total of 56 isolates were recovered.

The colonies of *E. tracheiphila* exhibited discernible morphological characteristics including a spherical appearance, featuring a smooth exterior bordered by neat margins. All isolates displayed a pale yellow or beige hue, with sporadic instances of coloration intensification following prolonged incubation periods. The colonies exhibited a silky and moist consistency, frequently manifesting a reflective or gleaming sheen. Significantly, the absence of translucency in the colonies hindered the transmission of light when subjected to an illumination source. These characteristics matched those documented in the literature for *E. tracheiphila* colonies (Rojas and Gleason, 2012; Mitchell and Hanks, 2009; De Mackiewicz *et al.*, 1998).

Biochemical tests revealed each isolate was test Gram-negative. All isolates tested positive for oxidase and catalase, confirming their existence. The tested isolates utilized citrate as a sole carbon source. Positive Methyl Red tests indicated mixed-acid fermentation. In contrast, Indole and Voges-Proskauer tests were negative for all isolates. Despite positive nitrate reduction tests, urease testing showed no production. The isolates' complete biochemical profiles corroborate the identification of *Erwinia tracheiphila*, revealing the behavioral traits of tested isolates.

Molecular Analysis of *E. tracheiphila*

The 16S rRNA gene of *Erwinia tracheiphila* was amplified using specific primer pairs, yielding bands of approximately 1500 bp, a characteristic size for *E. tracheiphila*, visible on a 1% agarose gel stained with ethidium bromide (Figure 1). Subsequent phylogenetic analysis of DNA sequences from highly virulent *E. tracheiphila* isolates (B.W 7, B.W 10, B.W 12, B.W 19, B.W 26, B.W 33, B.W 36, B.W 37, B.W 40, B.W 41, B.W 43, B.W 50, and B.W 56), compared to reference DNA sequences from the National Center for Biotechnology Information (NCBI) database, revealed a significant similarity ranging from 99% to 100% with previously submitted sequences from China and India (Table 1).

These results confirmed the initial hypothesis that the pathogenic bacterial isolates responsible for cucumber bacterial wilt disease in Pakistan belong to the *E. tracheiphila* (Figure 2). This finding contributes significantly to our understanding of the genetic variability and geographical distribution of *E. tracheiphila* in Pakistan.

Pathogenicity Confirmation

Pathogenicity tests performed on cucumber seedlings of four varieties viz. Kheera local, Punjab Kheera, Yousaf F1, and Adventa 703 revealed each isolate exhibited variations in the appearance of symptoms on different

plants. Symptoms appeared on plants within 10 days of inoculation and were marked as having the highest level of virulence, three plus signs (+++); moderate virulence (two plus signs (++); mild virulence (one plus sign (+); and no sign of disease appeared until day 21 as not virulent (-). Among 56 isolates tested against *E. tracheiphila* infection, 14 isolates were appeared highly virulent, 15 virulent, 14 mild virulent and 13 nonvirulent. With few exceptions, the same trend was observed for other cucumber varieties tested (Table 2).

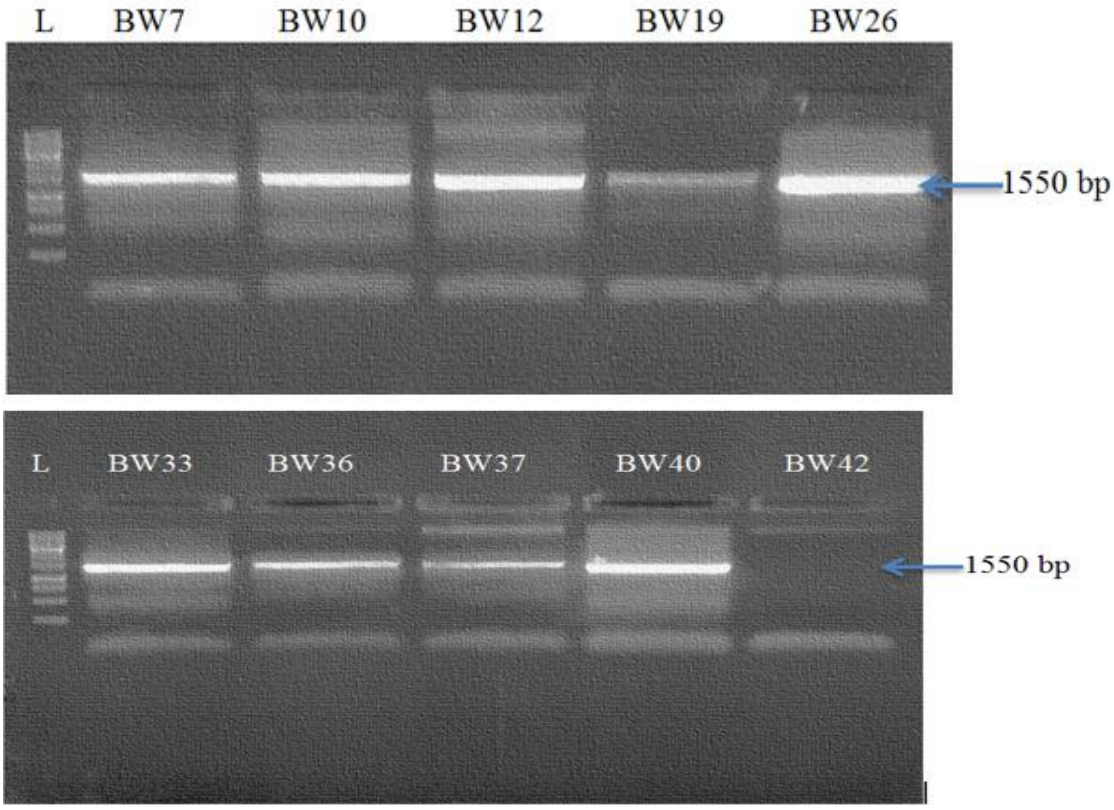

Figure 1. PCR amplification of 16S rRNA gene of *Erwinia tracheiphila*.

Table 1. Accession numbers and similarity index of *E. tracheiphila* isolates on the bases of 16s rRNA gene.

Isolate Name	Accession No.	Gene	Similarity %	Reference Isolate
B.W 7	OR041519	16s RNA	100	EU490598
B.W 10	OR041590	16s RNA	99	NR044924
B.W 12	OR041591	16s RNA	100	MK356446
B.W 19	OR046070	16s RNA	100	MK356442
B.W 26	OR046071	16s RNA	99	MK356441
B.W 33	OR041654	16s RNA	100	CP013970
B.W 36	OR041657	16s RNA	99	MK356446
B.W 37	OR041659	16s RNA	99	MN620381
B.W 40	OR041660	16s RNA	100	CP089934
B.W 41	OR041675	16s RNA	100	MT760003
B.W 43	OR041508	16s RNA	99	MK356446
B.W 50	OR041507	16s RNA	99	MT760003
B.W 56	OR041504	16s RNA	100	MN620381

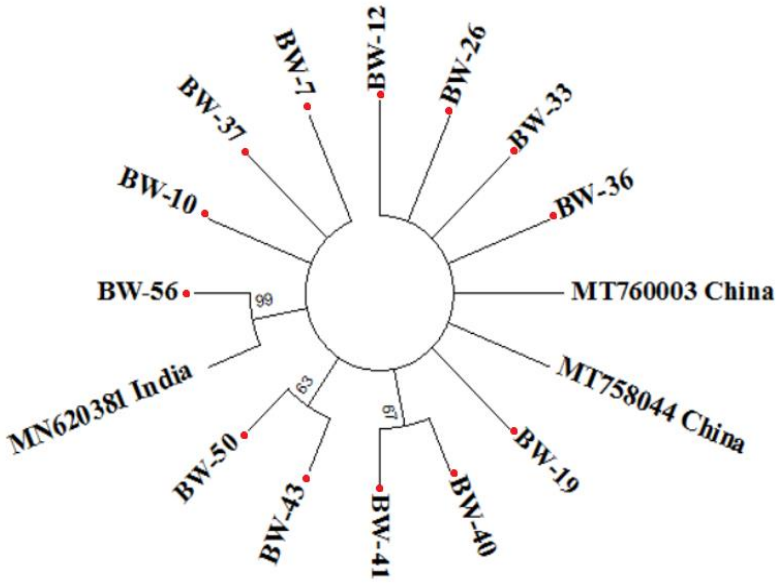

Figure 2. Phylogenetic homology between isolates of *E. tracheiphila* and its closest relatives based on phylogenetic analysis of 16S rRNA gene.

Table 2. Virulence patterns of different *E. tracheiphila* isolates on cucumber cultivars.

B.W	KL	PK	YF1	A703	B.W	KL	PK	YF1	A703	B.W	KL	PK	YF1	A703
B.W 1	-	-	+	+	B.W 20	-	-	-	-	B.W 39	+++	+	++	+
B.W 2	+	-	+	+	B.W 21	-	-	+	-	B.W 40	+++	+++	+++	+++
B.W 3	-	+	-	-	B.W 22	+	++	+	++	B.W 41	+++	+++	+++	+++
B.W 4	-	-	+	-	B.W 23	-	-	-	-	B.W 42	++	+++	++	+
B.W 5	++	++	+++	+++	B.W 24	+	+	-	+	B.W 43	+++	+++	+++	+++
B.W 6	+	-	+	-	B.W 25	++	+++	+	+	B.W 44	+	+	-	+
B.W 7	+++	+++	+++	+++	B.W 26	+++	+++	+++	+++	B.W 45	-	-	-	-
B.W 8	-	-	-	-	B.W 27	++	+	-	+++	B.W 46	+	-	+	-
B.W 9	++	+++	++	+	B.W 28	++	+++	-	++	B.W 47	++	++	+++	+
B.W 10	+++	+++	+++	+++	B.W 29	-	+	+	-	B.W 48	+	+	-	+
B.W 11	++	++	+++	++	B.W 30	+	-	+	-	B.W 49	-	+	-	-
B.W 12	+++	+++	+++	+++	B.W 31	+	+	-	+	B.W 50	+++	+++	+++	+++
B.W 13	++	++	++	++	B.W 32	-	-	-	-	B.W 51	++	+++	++	+
B.W 14	+	++	+++	+	B.W 33	+++	+++	+++	+++	B.W 52	++	++	++	+++
B.W 15	++	+	++	++	B.W 34	+	+++	++	++	B.W 53	-	-	-	-
B.W 16	+	+	+	-	B.W 35	++	+	+	++	B.W 54	-	-	-	-
B.W 17	+	++	+	++	B.W 36	+++	+++	+++	+++	B.W 55	++	+	+++	-
B.W 18	+	+	+	+	B.W 37	+++	+++	+++	+++	B.W 56	+++	+++	+++	+++
B.W 19	+++	+++	+++	+++	B.W 38	++	++	+++	+++					

B.W = Bacterial Wild Isolate, KL = Kheera Local, PK = Pakistan Kheera, YF1 = Yousaf F1, A703 = Adventa 703.

Rhizobacterial Compatibility Test

Compatibility test of the rhizobacterial isolates revealed *P. stutzeri*-2 exhibited an inhibition zone of 5.23 mm against *B. subtilis*-1 and 9.65 mm against *P. fluorescens*-3. *B. safensis*-2, on the other hand, displayed a 4.45 mm inhibition zone against *P. putida*-5. All the remaining isolates exhibited similar growth on the plate, showing no signs of incompatibility with each other (Table 3).

In Vitro* Efficacy of Rhizobacterias against *E. tracheiphila

Among all the tested treatments, *Pseudomonas fluorescens*-3 demonstrated the most significant growth inhibition (39.04 mm) against the pathogenic bacteria B.W7. Following closely were *Bacillus subtilis*-1 (38.14 mm) and *Pseudomonas putida*-5 (37.63 mm) (Table 4). In contrast, the control treatments showed no growth inhibition of the pathogenic bacteria. The experiment results highlight the substantial efficacy of *P. fluorescens*-3 rhizobacteria against all tested pathogenic isolates of *E. tracheiphila*. Subsequent effectiveness was observed in *P. putida*-5 and *B. subtilis*-1, particularly against B.W7, B.W12, B.W26, B.W33, B.W36, B.W43, and B.W56. These findings suggest that *P. fluorescens*-3, *P. putida*-5, and *B. subtilis*-1 showed promising results as biocontrol agents against *E. tracheiphila* (Figure 3).

Table 3. Evaluation of self-compatibility of selected PGPR strains.

Strain	Pf-3	Pu-5	Ps-2	Bs-1	Bs-2	Ps-1
Pf-3	✓	✓	×	✓	✓	✓
Pu-5	✓	✓	✓	✓	×	✓
Ps-2	×	✓	✓	×	✓	✓
Bs-1	✓	✓	×	✓	✓	✓
Bs-2	✓	×	✓	✓	✓	✓
Ps-1	✓	✓	✓	✓	✓	✓

Note: ✓ = compatible (no inhibition zone appear); × = incompatible (inhibition zone appear).

Table 4. *In vitro* assessment of selected rhizobacterial efficacy against *E. tracheiphila*.

Isolate	Mean growth inhibition (mm)					
	Pu-5	Pf-3	Ps-2	Bs-1	Bs-2	Ps-1
B.W7	37.637A	37.667A	18.377B	38.137A	20.923CD	39.04A
B.W12	35.283AB	22.607C	15.32BC	19.33C	17.93E	34.93B
B.W26	33.167AB	31.773B	25.193A	35.26AB	21.96C	19.95DE
B.W33	36.013AB	36.46A	25.64A	17.43C	37.31A	17.12E
B.W36	25.897C	24.517C	9.9867D	36.78AB	18.78DE	19.89DE
B.W43	19.82D	35.087AB	11.9CD	21.36C	21.1CD	20.87D
B.W56	30.823BC	34.317AB	25.89A	31.99B	25.19B	24.52C
Control	0E	0D	0E	0D	0F	0F
Grand Mean	27.330	27.803	16.539	28.613	20.399	22.040
CV	2.06	7.27	13.71	12.33	8.32	8.92

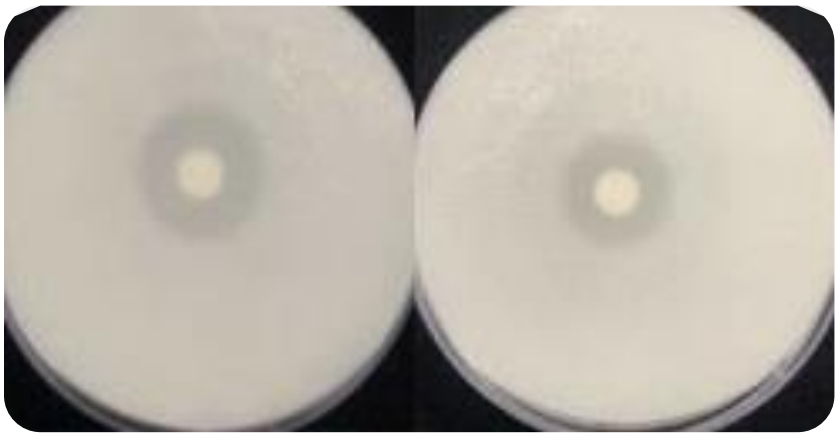

Figure 3. Evaluation of rhizobacterial inhibition zones against *E. tracheiphila*.

Biological Control

The results of the biological control experiment revealed a significant difference among treatments in various parameters. Vine length of the treated plants showed a statistically significant disparity between treatment means, with T6 exhibiting the highest vine length, 180% longer than the positive control and 29% longer than the negative control. T7 was statistically at par with T6, followed by T8. Treatments T7 and T6 gave the higher number of branches (168% greater than the positive control and 62% greater than the negative control), followed by T8. There was a statistically significant variation observed in the number of leaves per plant for different treatments. T6 exhibited the greatest leaf production, with a 140% increase in comparison to the positive control and a 42% increase over the negative control. T6 and T7 exhibited statistical similarity, with T8. Application with T6 revealed the increase in leaf length, 81%, in comparison to the positive control and 19% in comparison to the negative control. T6 and T7 exhibited statistical similarity, with T8 for increased leaf length. A similar trend was observed for leaf width where T6 outperformed all other treatments, measuring 62% increase compared to positive control and 11% to negative control. The performance of T6 and T7 were statistically same with T8 following suit. Plant vigor also varied significantly between treatment means. The vigor of T1 was the highest at 84.37%, in comparison to the control treatments (Table 5).

The final data component pertains to the severity of bacterial wilt. The results show that positive control treatment (T2) expressed the highest level of bacterial wilt severity, which was 100% greater than that of negative control treatment (T1), in which no pathogenic bacteria were inoculated. T7 treatment had the lowest bacterial wilt severity (52.1%) and is considered the most effective treatment against bacterial wilt of cucumber when a pathogen attacks the crop. The efficacy of this treatment was found to be statistically equivalent to that of T5, and T8 followed these treatments.

Table 5. Differential effects of biological control treatments on growth and disease parameters.

Treatment	Vine Length	Number of branches	Number of leaves	Leaves length	Leaves width	Vigor %	Wilt severity
T1	67.513 BC	2.6250 CD	16.125 BCD	10.338 B	11.813A	84.375A	0.0000D
T2	31.138 E	1.6250 D	9.2500 F	6.7875 C	8.1125B	0.0000 E	100.00A
T3	61.875 CD	3.0000 BC	15.750 CD	10.863 AB	11.963A	22.500D	81.350AB
T4	59.775 CD	2.8750 BC	14.250 DE	11.475 AB	12.188A	28.125CD	74.300BC
T5	49.713 D	2.1250 CD	11.625 EF	10.538 AB	11.738A	25.625D	78.975AB
T6	87.200 A	4.1250 A	23.000 A	12.300 A	13.187A	49.375BC	55.375C
T7	82.262 AB	4.2500 A	19.750 AB	11.462 AB	12.600A	51.250B	52.125C
T8	80.587 AB	3.8750 AB	19.250 BC	AB	12.400A	48.750BC	60.250BC

It is evident from the results that strain combinations *P. flurescens*-3 (Pf-3), *P. putida*-5 (Pu-5) showed the greatest induced resistance against bacterial wilt disease of cucumber. Other PGPR treatments were also found to reduce the severity of bacterial wilt, as compared to the positive control. The observed symptomatology in plants treated with PGPR treatments varied between 52% and 81%, whereas the disease control treatment exhibited 100%. The findings suggest that a combination of two strains of PGPR (*P. flurescens*-3, *P. putida*-5) should be subjected to further assessment in field trials.

Integrated Disease Management

The results of the integrated disease management experiment revealed T8 had the highest vine length, branches per plant, and leaf length. T8 also had the longest recorded leave length, with a 65% increase compared to the positive control and 21% more than the negative control. T6 had the highest leaf width, 63% greater than the positive control and 13% more than the negative control. T1 had the highest plant vigor (85%), while T2 showed 0%. T8 showed the best results of 55.62% plant vigor in the presence of pathogenic bacteria. T8 was found to be statistically equivalent to T6 and T7. These findings suggest that the combination of *P. putida*-5 and *B. subtilis*-1 along with the chemical Airone showed the greatest induced resistance against bacterial wilt disease in cucumber plants. Other treatments also reduced the severity of bacterial wilt in some plants compared to the positive control. The observed symptomatology in plants treated with PGPR

along with the chemical varied between 42% and 63%, whereas the disease control treatment exhibited 100%. The findings suggest that a combination of two strains of PGPR (*P. putida*-5, *B. subtilis*-1) along with systemic bactericides Airone gave best results in reducing *E. tracheiphila* infection in cucumber (Table 6).

Table 6. Evaluation of integrated disease management for growth efficacy and bacterial wilt disease severity.

Treatment	Vine Length	Number of branches	Number of leaves	Leaves length	Leaves width	Vigor %	Wilt severity
T1	65.575 AB	2.3750 AB	17.375 A	10.320 B	11.688 A	85.000 A	0.0000 C
T2	27.387 C	1.1250 C	9.6250 B	7.5500 C	8.0750 B	0.0000 D	100.00 A
T3	53.575 AB	2.1250 B	15.375 A	10.988 AB	11.538 A	33.125 BC	63.375 B
T4	54.875 AB	2.3750 AB	15.500 A	11.375 AB	12.038 A	38.750 BC	60.250 B
T5	49.938 B	2.0000 BC	14.625 A	11.075 AB	11.663 A	30.000 C	68.875 B
T6	63.888 AB	2.5000 AB	16.750 A	12.413 A	13.225 A	48.125 BC	50.000 B
T7	62.313 AB	2.5000 AB	16.750 A	12.038 AB	12.688 A	46.875 BC	52.375 B
T8	70.025 A	3.1250 A	18.250 A	12.513 A	12.888 A	55.625 B	42.125 B

DISCUSSION

Cucumbers (*Cucumis sativus)* are a nutritious vegetable that provide a range of vitamins and minerals while being low in calories. In Pakistan, cucumber is widely cultivated in various regions for both local consumption and export. Bacterial wilt of cucurbits, also known as cucumber wilt, is a devastating plant disease that affects cucumber, melon, and other cucurbit crops. The causative agent of this disease is the bacterium *Erwinia tracheiphila.* Latin (2000), Rojas *et al.* (2011), and Smith (1911) reported significant production and quality decreases in all cucurbit crops, including cucumber, in the United States due to major epidemics produced by the bacteria *E. tracheiphila*. In present studies, a total of 56 *E. tracheiphila* isolates were recovered from the symptomatic plant portions among which, 13 isolates were tested highly virulent. These results are consistent with the observations made by Smith (1911), who identified *E. tracheiphila* as a significant constraint in cucurbit production, including cucumber, due to its role as a primary host of bacterial pathogens. Smith (1911) also reported that early wilting caused by *E. tracheiphila* can lead to yield losses ranging from 80% to 100%. The results of Zehnder *et al.* (1997) indicate that bacterial wilt is a significant cause of crop damage in various cucurbit crops, particularly in the genera *Cucurbita* and *Cucumis*. Sherf and MacNab (1986) stated in 1986 that muskmelon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) are two of the most vulnerable crops, with economic losses of cucurbit crops from bacterial wilt exceeding 75%. The findings of several biochemical identification tests performed on the 13 highly virulent isolates indicated that all of the isolates obtained were members of *the Erwinia tracheiphila* family, as described by Sarkar and Chaudhuri (2016). The genetic analysis of 16S rRNA genes through polymerase chain reaction (PCR) has allowed for the molecular identification of these highly virulent strains of *E. tracheiphila*. This finding is significant for providing an understanding of the pathogenic bacterium's genetic diversity and its distribution across different regions. Sequence analysis was conducted on the amplified product using Basic Local Alignment Standard Tools (BLAST). The results of this study showed that the sequences obtained were extremely similar (98-100% similarity) to previously submitted sequences of the target genes from China and India, proving that the isolates of pathogenic bacteria associated with bacterial wilt disease of cucumber obtained from study location in Pakistan. A GenBank Blast Search of publicly available bacterial databases revealed that 1550 bp of 16S rRNA region exactly matched with *E. tracheiphila* data base (assigned accession number OR041519, OR041591, OR046070, OR041654, OR041660, OR041675, and OR041504) with 100% similarity and sequentially 99% (assigned accession number OR041590, OR046071, OR041657, OR041659, OR041508, OR041507). Results of 16s rRNA gene amplification are supported by the findings of Pattanayak, 2021. The findings suggest that *E. tracheiphila* has a broad distribution across various geographic regions and has the potential to cause bacterial wilt disease outbreaks due to its ability to spread quickly. This is supported by the significant genetic similarity observed among the isolates from

different regions. The present study's findings are in line with prior research that has documented the worldwide prevalence of this *E. tracheiphila* and its capacity to inflict significant economic damage on cucumber crops (Shapiro *et al.*, 2018).

The best antagonistic plant growth-promoting rhizobacteria against *Erwinia tracheiphila* was chosen through an experiment from among the six PGPRs that existed already in the bacteriology lab. The study's findings indicate that *Pseudomonas fluorescens*-3 (Pf-3), *Pseudomonas putida* (Pu-5), and *Bacillus subtilis*-1 (Bs-1) demonstrated notable effectiveness against all examined pathogenic isolates of *E. tracheiphila*. The results of the research indicate that Bs-1, Pu-5, and Pf-3 exhibited notable growth inhibition against the pathogenic bacteria. Additionally, *Pseudomonas fluorescens*-1 (Pf-1) demonstrated a relatively high level of growth inhibition, ranking second among the tested PGPRs. The study's findings suggest that Pf-3, Pu-5, and Bs-1 have the potential as effective biocontrol agents against *E. tracheiphila*. The present study's findings are consistent with those of Roberts *et al.* (2018), which suggest that *Pseudomonas fluorescens* can serve as a biocontrol agent for *E. tracheiphila*. According to Zehnder *et al.* (1997) findings, *Bacillus subtilis* exhibits antagonistic properties toward *E. tracheiphila*.

The findings of the compatibility examination indicate that particular interactions occurred among the distinct rhizobacterial isolates. The results of the study indicate that *P. stutzeri*-2 (Ps-2) exhibited inhibition zones against *B. subtilis*-1 (Bs-1) and *P. fluorescens*-3 (Pf-3), whereas *B. safensis*-2 (Bs-2) displayed an inhibition zone against *P. putida*-5 (Pu-5). The findings indicate that the co-inoculation of these specific bacterial strains may not be feasible due to potential incompatibility. The compatibility of *P. putida*-5 and *P. fluorescens*-3, rhizobacteria strains has been examined and the results indicate that they are compatible with each other. These findings align with the research conducted by Farhan *et al.* (2010), on the compatibility of *P. putida*-5 and *P. fluorescens*-3. In a study that was conducted by Devi and Prakasam (2013), it was found that *P. fluorescens*-3 and *B. subtilis*-1 exhibit a high level of compatibility with each other and are unable to coexist in terms of growth.

Biological control experiments were conducted to demonstrate the induction of systemic resistance in cucumber plants against bacterial wilt disease, as well as the resulting plant growth promotion from PGPR. Singh (2015) asserts that the natural promotion of plant growth has the potential to reduce the incidence of disease and pests while simultaneously enhancing plant yields. The results of the experiments indicate that the use of PGPR had a significant positive impact on the primary growth characteristics of cucumber plants. These findings are consistent with previous research conducted by De Vleesschauwer and Höfte (2009), which suggests that the biological agents used in the treatments can stimulate plant growth through hormonal production and induce systemic resistance in plants, providing protection against range of pathogens. Other studies, such as Jayaraj and Alleyne (2015) research on biological control agents' formulation for controlling sustainable disease, also support these findings. The results of our study indicate that the majority of PGPR treatments had a significant ($P < 0.05$) positive impact on the overall plant vigor of cucumber plants. when compared to the negative control treatment. In T6 indicates that the combination of *P. flurescens* & *P. putida* enhances all growth parameters even in the presence of pathogenic bacteria. This observation is consistent with the results of prior research carried out by Rekha *et al.* (2007). In the study T7, a combination of *P. flurescens* & *B. subtilis* was found to effectively suppress the disease by 52.1% when compared to the control treatment. The findings are consistent with prior research carried out by Al-Fadhal *et al.* (2019) in Iraq, where it was observed that *Pseudomonas fluorescens* and *Bacillus subtilis* acts as the most effective antagonist against damping off disease of cucumber. According to the research findings of Zehnder *et al.* (1997)**,** *Bacillus subtilis* has been identified as the most efficient biological control measure against *E. tracheiphila*. The compatibility between both strains is determined, so it enhances their effectiveness in triggering systemic resistance against bacterial wilt in cucumber plants.

The results of the integrated disease management experiment suggest that combination *P. putida*-5, *B. subtilis*-1 and systemic bactericides, Airone gave best results in reducing *E. tracheiphila* infection in cucumber. Integrated disease management (IDM) strategies involve the coordinated use of multiple strategies to ensure more yields of the crops while minimizing pathogen damage to levels below the economic threshold. The present investigation involved the development of integrated disease management (IDM) strategies that

incorporated chemical and biocontrol approaches to reduce the occurrence of bacterial wilt diseases in cucumbers. Despite the utilization of a range of systemic and non-systemic fungicides for the purpose of managing bacterial wilt disease, the efficacy of disease control in fields remains unsatisfactory due to a number of environmental factors and the development of resistance in pathogens toward chemicals that have been consistently used by Thind *et al.* (1991). The present investigation demonstrated that treatments, specifically T8 (combination of *P. putida*-5 & *B. subtilis*-1 + Airone) and T7 (combination of *P. flurescens*-3 & *B. subtilis*-1 + Airone) applied to cucumber, exhibited remarkable efficacy in minimizing disease incidences. The combination of different treatments that may induce beneficial plant responses and a favourable condition for plant growth led to the outcomes seen in the IDM treatment Dhawan and Peshin (2009). This approach has been recognized for its cost-effectiveness and environmentally friendly nature (Gupta *et al.*, 2021).

CONCLUSION

The findings of this study indicate that the implementation of integrated disease management (IDM), which involves the use of targeted treatments obtained from both biological and chemical methods, can be an effective strategy for reducing the severity and incidence of bacterial wilt disease in cucumber plants.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

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