

RESEARCH

Open Access



Influence of *Pseudomonas fluorescens* mutants produced by transposon mutagenesis on in vitro and in vivo biocontrol and plant growth promotion

Abdullah S. Alsohim

Abstract

The fitness of microbes and their colonization efficiency in plants is significant for promotion of plant growth, but the mechanism between plants and bacteria in rhizospheric region is not clearly explained. This study focused on identification and characterization of some plant growth promoting biocontrol bacteria. A total of 94 bacteria strains were isolated and tested for different plant growth promotion activities and their antagonistic behaviors towards different pathogenic fungi. The isolated bacteria were categorized into 23 bacterial genera, using 16S rRNA. The most predominant genera of bacterial isolates were *Pseudomonas* (4 species) and *Bacillus* (6 species). The isolates (Qassim University Saudi Arabia (QUSA) 52 and 45) and their transposon mutagenesis mutants inhibited mycelium growth of *Rhizoctonia solani*, *Alternaria* sp., and *Colletotrichum* sp. Isolates 52 and 45 of *P. fluorescens* and their mutants' 52-M12, 45-M19, and 45-M20 yielded the highest dry weight and shoot, and root length in alfalfa plants. Furthermore, the efficiency of these bacterial isolates and mutants against *R. solani* was considerably higher than the control treatment.

Therefore, application of biocontrol agents can significantly control the soil-born fungal pathogen in alfalfa plants.

Keywords: Plant growth-promoting rhizobacteria, *Pseudomonas* sp., *Bacillus*, Mutagenesis, Bio-control, Alfalfa

Background

Numerous biocontrol bacterial species are still unknown and the knowledge of their antagonism against pathogenic fungi is still poor. Thus, the accurate and reliable identification of biocontrol bacteria is an important task for many, if not all disciplines within microbiology (Busse et al. 1996). Traditionally, biocontrol bacterial isolates were identified and classified based on phenotypic methods according to morphological, physiological, and metabolic markers (Rosselló-Mora and Amann 2001). There are major drawbacks to these phenotypic approaches. They are time-consuming, tedious, and the variability among strains belonging to the same species

can be worth (Busse et al. 1996; Mignard and Flandrois 2006). The diversity of rhizobia isolated from *Phaseolus vulgaris* has been examined worldwide, using a variety of techniques and criteria, where beans originated (Central and South America) and where it was introduced (Koskey et al. 2018). The PCR–RFLP analysis of the 16S–23S rDNA intergenic transcribed spacer (ITS) has proven to be a useful tool for studying the genetic diversity of rhizobia belonging to different genera (Vessey and Chemignigwa 2006, and Lin et al. 2007).

Sequencing of the 16S rRNA gene is a genotypic method by which bacteria can be classified and identified. In literature, there are various reasons that 16S rRNA is the most commonly used genetic marker for bacterial identification and classification (Janda and Abbott 2007). These reasons are (1) its function has not

Correspondence: a.alsohim@qu.edu.sa; alsohim@hotmail.com
Plant Production and Protection Department, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, Saudi Arabia

changed over time, (2) the 16S rRNA gene is universal in bacteria, and (3) it is large enough for informatics purposes. Therefore, the advantage of using 16S rRNA over phenotypic approach was reported because it does

not require optimal growth conditions and/or cultivation of microorganisms. The 16S rRNA gene sequence has been used to identify various types of slow- and fast-growth bacteria and rare as well as novel species of

Table 1 Sources and locations of bacterial isolates were collected from Qassim region in Saudi Arabia/QUSA

QUSA	Sample	Source
1	Soil	Saxaul/Ghada tree (<i>Haloxylonpersicum</i>) - Rawdat Al-Tanhat
2	Soil	Arta shrub (<i>Calligonumcomosum</i>) - Rawdat Al-Tanhat
3, 4, 20	Root	
21	Leaves	
5-7	Soil	Carrot - Qassim University farm
8, 22	Leaves	Damas tree (<i>Conocarpus</i>) - Qassim University
9-12	Leaves	<i>Albizia lebbeck</i> tree - Qassim University
13-15	Leaves	Palm tree/ <i>Phoenix dactylifera</i> - Al-Rajhi palm orchards, Buraidah
16-19	Leaves	Ivy/ <i>Convolvulus</i> - Al-Rajhi palm orchards, Buraidah
23-27	Soil	Barley - Rawdat Al-Tanhat
28	Roots	
29, 30	Soil	Chenopodiaceae grass - Rawdat Al-Tanhat
31, 32	Roots	
33-34	Leaves	<i>Rhazya stricta</i> /Al-Harmal - Rawdat Al-Tanhat
35	Soil	
36	Leaves	<i>Plantago ovate</i> /Al-Riblaa - Rawdat Al-Tanhat
37-39	Roots	
40	Soil	
41-43	Roots	<i>Launaea</i> (Al-Bakraa) - Rawdat Al-Tanhat
44-50	Soil	Clover - Buraidah
51, 52	Roots	
53	Roots	Wheat - Buraidah
54	Leaves	Undefined grass 1 - Buraidah
55-58	Roots	
59-65, 80	Soil	
66	Roots	<i>Pulicaria crista</i> /Arfag - Buraidah
67-69	Roots	<i>Heliotropium bacciferum</i> - Buraidah
70, 71	Soil	
72	Leaves	
73, 74	Leaves	<i>Astragalus spinosus</i> /Al-Katad - Buraidah
75, 77	Soil	
76, 78	Roots	
79	Soil	<i>Pulicaria crista</i> - Buraidah
81	Soil	Clover - Almulaida, Buraidah
82	Soil	Arta tree - Almulaida, Buraidah
83-84, 94	Roots	Undefined grass 2 - Buraidah
85	Soil	
86-87	Leaves	Undefined grass 3 - Buraidah
88, 89, 90	Soil	
91-93	Roots	Undefined grass 4 - Buraidah

bacteria (Alsohim et al. 2014 and Mignard and Flandrois 2006).

In Saudi Arabia, commercialization of microbial bio-fertilizers and biocontrol agents are still in its early stage, which no local plant growth promoting rhizobacteria (PGPR) are available in the market (Alsohim et al. 2018). To the best of our knowledge, no study has been performed to develop effective local microbial strains. Thus, the most microbial bio-fertilizers and biocontrol agents in the market are imported from other countries. It has been shown that the survivability of PGPR isolated from a temperate climate rapidly decreases when added to arid soil (Bhattacharjee et al. 2008). Isolation of native strains adapted to the arid environment may contribute to the formulation of inoculants suitable for use with local regional crops. Native isolates may be preferred in the selection of bacteria for inoculation, as they are adapted to the environment and thus can be more competent than imported biocontrol bacteria (Karagöz et al. 2012). Using natural soil isolates inoculated into the plant rhizosphere has the advantage of being the easiest way for adaptation and succession (Chen et al. 2006). *Pseudomonas* bacteria are used as model organisms in these colonization studies because they are among the important bacteria existing in the rhizosphere (Lugtenberg et al. 2001). For example, Lugtenberg et al. (2001) conducted a systematic search for mutants affected in competitive root colonization with *P. fluorescens* WCS365. This was done via transposon (Tn5) mutagenesis, and they generated a bank of mutants that were used to test their ability of in competitive fitness. It also has been reported that bacteria (*Pseudomonas fluorescens*) colonized the base of the root in higher densities than in other parts of the root and that they decreased by twofold towards the top of root (Simons et al. 1996 and Chin-A-Woeng et al. 1997). The Qassim region, Saudi Arabia, is located in an arid zone characterized by low rainfall, extreme temperatures, and infertile with salt-affected soils. However, agricultural activities in Qassim are known to depend on groundwater and the use of chemical fertilizers to enhance soil fertility and crop productivity. The excessive use of chemical fertilizers often results in unexpected harmful environmental effects, including accumulation of nitrate in plant tissues, leaching of nitrate into groundwater and surface runoff of phosphorus and nitrogen. Obviously, an integrated nutrient management system is required to maintain agricultural productivity and protect the environment.

Therefore, microbial inoculants are promising components for integrated solutions to agro-environmental problems (Alsohim 2015), which deduced that PGPR isolates (*Sinorhizobium* sp.) may have the potential to act as PGPR that can enhance plant growth, particularly under stressed environmental conditions. It is, therefore,

necessary to develop effective biofertilizers containing native PGPR strains that can adapt well to the arid environment. Rhizosphere of perennial plants, which are well adapted to the local environment are perhaps the best source of native microbial isolates (Hanna et al. 2012).

Therefore, the objectives of this study were to (1) isolate and purify biocontrol bacteria from a range of plant species, (2) use mutagenesis to identify mutants that promote the plant growth, in vitro evaluation of bacteria and mutants for suppression of oomycete and fungal growth together, and (3) characterize the selected bacteria by genome sequencing and conduct in vivo test with those bacteria mutants for plant growth promotion with and without fungal pathogens.

Material and methods

Isolation of bacteria strains

Bacterial isolates were obtained from plant samples of various trees, bushes, and desert grasses growing at different sites in the Al-Qassim region, Saudi Arabia (Alsohim 2015) (Table 1). The plant samples contained some parts of soil, roots, and leaves, were carefully collected in plastic bags, and then stored at 4 °C until used.

After removing adhering soil, the plant root samples were cut into small pieces and crushed in sterile water. The plant leaves were also crushed in sterile water. As well as, 10 g of the soil from each of the rhizosphere samples was transferred to 100 ml of sterile water. These 3 suspensions were serially diluted, and 100 µl of each 10⁻⁴, 10⁻⁵, and 10⁻⁶ homogenized dilutions was distributed into nutrient agar (NA) medium. Plates were

Table 2 Plant pathogenic fungi tested in the fungal inhibition growth assay and their sources

No.	Fungi	Source
1	<i>Fusarium moniliform</i>	Drought on palm tree - pathology lab
2	<i>Fusarium solani</i>	Biotechnology lab
3	<i>Fusarium oxysporum</i>	Biotechnology lab
4	<i>Fusarium graminearum</i>	Pathology lab
5	<i>Fusarium oxysporum.c.</i>	Pathology lab
6	<i>Fusarium</i> sp. (purple)	Pathology lab
7	<i>Fusarium</i> sp. (white)	Isolated from tomato - pathology lab
12	<i>Fusarium</i> sp. (white1)	Palm tree - pathology lab
8	<i>Fusarium</i> sp. (pink)	Biotechnology lab
9	<i>Rhizoctonia solani</i>	Biotechnology lab
10	<i>Colletotrichum</i> sp.	Al-Rajhi horticulture - pathology lab
11	<i>Botrytis</i> sp.	Strawberry fruit
13	<i>Alternaria</i> sp.	Leaf spot on palm tree - pathology lab
14	<i>Stemphylium</i> sp.	Pathology lab

Table 3 Identification of bacterial isolates (QUSA) and their high similarity using 16S rDNA gene sequencing

QUSA	Description	Identity
2	<i>Bacillus</i> sp. PVR-YHB-4-1	97%
5	<i>Klebsiella</i> sp. IPPW-15	93%
7	<i>Chryseobacterium</i> sp. K-2	99%
10	<i>Acinetobacter</i> sp. NC95 partial	99%
11	<i>Bacillus licheniformis</i> strain MK7	98%
14	Uncultured <i>Acinetobacter</i> sp. clone GI3-S-1-G03	99%
16	<i>Psychrobacter</i> sp. MVS1-N6	98%
17	<i>Pseudomonas</i> sp. CB14	88%
19	<i>Paracoccus</i> sp. B22	98%
23	<i>Microbacterium</i> sp. AHW1G3	92%
25	<i>Pseudomonas stutzeri</i> strain L1	99%
26	<i>Pantoea dispersa</i> strain p91_G09	98%
27	Uncultured <i>Pseudomonas</i> sp. clone CV-3	93%
28	<i>Stenotrophomonas maltophilia</i> strain Ysm	82%
29	<i>Lysinibacillus fusiformis</i> strain B-13	93%
30	<i>Enterobacter</i> sp. FCB1	98%
31	<i>Pseudomonas</i> sp. NR 6-02	89%
32	<i>Pseudomonas syringae</i> strain 8532	98%
33	<i>Achromobacter</i> sp. GMCo22	86%
34	<i>Pantoea</i> sp. LP10_RH04	99%
35	<i>Stenotrophomonas</i> sp. SMAKK001	85%
36	<i>Bacillus subtilis</i> strain PSM5	85%
37	<i>Bacillus</i> sp. RC2 partial	86%
40	<i>Arthrobacter nicotianae</i> strain Lb-41	91%
41	<i>Pseudomonas</i> sp. AD3.12	99%
42	<i>Cronobacter dublinensis</i> strain PYJ131	97%
43	<i>Pseudomonas</i> sp. TK37	98%
44	<i>Pseudomonas</i> sp. PS-4	98%
45	<i>Pseudomonas</i> sp. PS-4	99%
46	<i>Pseudomonas stutzeri</i> strain NM2E7	98%
47	<i>Exiguobacterium sibiricum</i> strain IHB B 14506	97%
48	<i>Pseudomonas stutzeri</i> strain S16(B)12 BRNEIST-DST	88%
49	<i>Bacillus</i> sp. T3-33	99%
51	<i>Pseudomonas amygdali</i> pv. <i>tabaci</i> strain YWTG-1	99%
52	<i>Pseudomonas fluorescens</i> Pf0-1, complete genome	98%
53	<i>Bacillus</i> sp. TAE13 gene	99%
57	<i>Rhodospirillum</i> sp. kmd_187	97%
58	<i>Pseudomonas</i> sp. TK37	99%
60	<i>Bacillus flexus</i> strain p8_B09	84%
61	<i>Bacillus</i> sp. 2BSG-MG-25 gene	99%
62	<i>Bacillus</i> sp. RSP-VIW-56	99%
63	<i>Pseudomonas</i> sp. CP07	89%
64	<i>Pseudomonas</i> sp. B3053	88%

Table 3 Identification of bacterial isolates (QUSA) and their high similarity using 16S rDNA gene sequencing (*Continued*)

QUSA	Description	Identity
65	<i>Planomicrobium</i> sp. PLB025	97%
66	<i>Pseudomonas</i> sp.H-128 strain H-128	94%
67	<i>Enterobacter aerogenes</i> strain DD2	93%
72	<i>Bacillus firmus</i> strain IARI-IR1	97%
74	<i>Bacillus licheniformis</i> strain SCK 121053	96%
77	<i>Mesorhizobium</i> sp. UASWS1017	98%
80	<i>Planococcus</i> sp. R-4-H-7	96%
86	<i>Bacillus atrophaeus</i> strain UAsDu82	96%
87	<i>Bacillus pumilus</i> strain K22	99%
90	<i>Salinicoccus hispanicus</i> strain JM-T11	99%
91	<i>Erwinia</i> sp. GY35 16S ribosomal RNA gene	99%
92	<i>Planomicrobium</i> sp. PLB025	99%

incubated at 28 ± 1 °C (Alsohim et al. 2018) for isolation of bacteria.

Identification of PGPR isolates through DNA sequencing

Rhizobacterial isolates, that grown overnight in nutrient broth and genomic DNA, were extracted by the hexadecyltrimethyl-ammonium bromide (CTAB) method (Doyle and Doyle 1990). The bacterial 16S rDNA primer Ps-for (5'GGTCTGAGAGGATGAT-CAGT3') and Ps-rev (5'TTAGCTCCACCTCGCGGG3') were used to amplify the 16S rDNA gene region. Polymerase chain reaction (PCR) was using a DNA Engine DYAD™ Peltier Thermal Cycler. Thermal cycling was performed by carrying out initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and the final extension at 72 °C for 8 min. The fragments obtained were analyzed via gel electrophoresis (24 × 12 cm) with 2% agarose, and carried out at 80 V for 2 h. A 1 kb DNA ladder load on the left lane of the gel was used as a molecular size marker. The PCR products were sent to the Macrogen Company in Korea for sequencing. The sequence search for the alignment of nucleotides was performed using the BLAST (basic local alignment search tool) web-based program.

Fungal inhibition growth assay

The bacterial isolates were tested for their ability to inhibit the growth of some important fungal plant pathogens. Four genera (*Fusarium*, *Rhizoctonia solani*, *Colletotrichum* sp., and *Botrytis* sp.) were tested in this assay. Fungal isolates information are described in Table 2. Four single-bacterial colonies of the bacterial isolates (*P. fluorescens*) were streaked on four edges of PDA containing Petri dishes and one disk of a new colony of the tested fungi was placed in the

Table 4 Reaction of mutants of bacterial isolate no. 52 (*Pseudomonas fluorescens*) tested for their antagonism to *Rhizoctonia solani* and *Colletotrichum* sp.

Mutant no.	<i>Rhizoctonia solani</i> after 1 day	<i>R. solani</i> after 5 days	<i>Colletotrichum</i> after 1 day	<i>Colletotrichum</i> after 5 days	Fluorescent
Iso. 52	+++	+++	++	++	+
1	+++	-	-	-	-
2	+++	+++	+++	+++	+
3	+++	+++	+++	+++	+
4	+++	+++	+++	+++	+
5	+	-	+	+	-
6	-	-	+++	+	-
7	-	-	-	-	-
8	+++	+++	+++	+++	-
9	+	+++	+++	+++	-
10	-	-	-	-	-
11	+++	+++	+++	+++	+
12	+	-	-	-	-
13	+++	+++	+++	+++	+
14	+++	+++	+++	+++	+
15	+	-	-	-	-
16	+	-	+	+	-
17	+++	+++	+++	+++	+
18	+	-	-	-	-
19	+++	+++	+++	+++	+
20	+	-	-	-	-
21	+	-	-	-	-
22	++++	++++	++++	++++ (wide free area)	+
23	+	-	+	-	-
24	++++	++++	++++	++++ (wide free area)	+
25		-		-	-
26		+++		+++	+
27		+++		+++	+
28		-		-	-
29		+++		+++	+
30		-		+	-
31		-		-	-
32		+++		+++	+

center of the Petri dish. The plates were incubated at 28 °C until the fungal mycelium covered the entire control dish after 4 days. The ability of IS-Ω-Km/hah mutants to inhibit the growth of the fungi was tested on PDA plates; all bacterial strains were inoculated as a 5 µl droplet from an overnight broth (cell density adjusted to OD 600 = 0.5) and allowed to grow overnight on a PDA agar plate. Fungi were then inoculated at the center of the PDA agar plates and incubated at 28 °C.

As the bacterial isolates showed antifungal activity, the observed inhibition of fungal mycelium growth was estimated using a three-level scale: (+) fungal mycelium showed delay in growth compared with the control, (++) fungal mycelium stopped growing in contact with the bacterial colony, and (+++) presence of clear antagonistic area free of fungal mycelium surrounding the bacterial colony. The inhibition zone (mm) was recorded by measuring the distance between the edges of the fungal mycelium and the bacterial streak.

Table 5 Quantitative reaction of mutants of bacterial isolate no. 45 (*Pseudomonas* sp.) tested for their antagonism against *Rhizoctonia solani*, *Colletotrichum* sp., *Fusarium solani*, *F. moniliforme*, *F. oxysporum*, *Fusarium* sp., and *Botrytis* sp.

Mutant No.	R. so	F. so	F. mon	F. ox	F. gra	Thial.	Bot.
Iso 45	+++	-	-	-	-	-	+++
1	++	-	-	-	-	-	+
2	-	-	-	-	-	-	+
3	-	-	-	-	-	-	+++
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	++
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	+++
10	-	-	-	-	-	-	++
11	-	-	-	-	-	-	+++
12	-	-	-	-	-	-	+
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	++
16	-	-	-	-	-	-	+++
17	-	-	-	-	-	-	+++
18	++	-	-	-	-	-	++
19	-	-	-	-	-	-	-
20	+++	-	-	-	-	-	+++

Construction of a *Pseudomonas fluorescens* transposon mutant using bacterial conjugation

Bacteria (*P. fluorescens*) isolates 45 and 52 were grown overnight in Luria broth (LB) at 27 °C and *Escherichia coli* was grown overnight in LB at 37 °C. Next, 900 µl of bacteria cells were mixed with 600 µl of the S17-1λpir

carrying plasmid pSCR001 (and 600 µl of *E. coli* strain containing the helper plasmid, DH5α (pRK2013), if used). The cells were mixed and centrifuged at 13,000 rpm to obtain a pellet. The mixture was then washed twice with sterile water. The cell suspension was spotted on LB agar plates and incubated overnight at 27 °C. These bacterial mating spots were then recovered and placed in 1 ml of sterile water and diluted 10-1 and 10-2. The suspensions were spread onto LB plates supplemented with antibiotics in order to select the trans-conjugants carrying the plasmid or for transposon mutagenesis. Two hundred isolates of IS-Ω-Km/hah transposon mutants were generated by the conjugation of bacteria with S17-1λpir (pSCR001) and selecting of Kmr mutants. Mutants were selected and arranged in micro titer dishes in order to produce frozen stocks and create the transposon library (Alsohim et al. 2014).

Testing selected bacteria in vivo for PGPR in the presence or absence of fungal isolates in greenhouse

In vivo experiments were conducted with biocontrol bacteria to determine whether they could improve plant growth (in the absence of pathogens) and protect plants (in the presence of pathogens). Alfalfa seeds were sterilized by Clorox 1% for 5 min and inoculated with selected bacterial isolates prior to exposure to the phytopathogenic fungi. The seeds were soaked for 10 min in bacterial containing bacterial isolates. The bacterial isolates (isolate 45; *Pseudomonas* sp., Mutant45-M19, Mutant 45-M20, isolate 52; *P. fluorescens* Pf0-1, Mutant 52-M12, and Mutant 52-M22) were tested for ability to protect the alfalfa roots against *Rhizoctonia solani*. The bacterial isolates (isolate 45; *Pseudomonas* sp., Mutant 45-M19, Mutant 45-M20, isolate 37; *Bacillus* sp.) were tested against *Botrytis* sp. The isolates were grown in nutrient broth on a rotary shaker at 30 °C and 170 rpm for 24 h.

Table 6 Thirty days after inoculation: shoot and root measurements of alfalfa plants under different treatments

No.	Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (mg/plant)	Dry weight (mg/plant)
1	Isolate 45	14.3 bc*	7.7 ba	1.4 c	0.6 b
2	Mutant 45-19	15.3 b	8.0 ba	1.5 b	0.7 ab
3	Mutant 45-20	16 b	8.0 ba	1.3 d	0.6 b
4	Isolate 37	20.7 a	8.7 a	1.6 a	0.8 a
5	Bot-45	16.3 b	7.0 ba	1.3 cd	0.7 ab
6	Bot-45-19	14.7 b	6.3 b	0.8 f	0.4 c
7	Bot-45-20	14.3 b	6.7 ba	0.6 g	0.3 c
8	Bot-37	16.7 b	7.7 ba	1.6 a	0.8 a
9	Bot.	0.0 d	0.0 c	0.0 h	0.0 d
10	Seeds	10.7 c	6.0 b	0.8 f	0.4 c

Seeds (Control 1) not inoculated with any microbe, Bot. (Control 2) only inoculated with *Botrytis* sp.

*Significant at the 0.01 probability level

The isolates were grown on LB broth at 28 °C for 3 days and further evaluated for their PGP traits. The suspensions were centrifuged in sterile 50-ml falcon tubes at 6000 rpm for 10 min. The pellets were then re-suspended in sterile distilled water to obtain a final concentration of 108 cells ml⁻¹, (OD = 0.5) at 600 nm, which was measured, using the viable plate count and optical density methods (Alsohim et al. 2018).

The soil used in the pot experiment was sterilized to minimize the influence of other microorganisms and air-dried prior to the experiment. Plants were monitored in a growth chamber at 30 °C with a photoperiod of 15 h. The experiments were arranged in a randomized complete block design (RCBD) with three replicates and repeated twice. After 4 weeks from inoculation, characteristics measured included plant root weight and length, plant shoot weight and length, and plant biomass (wet and dry).

Statistical analysis

The analysis of variance for plant characteristics was computed, using the MSTATC microcomputer program (MSTATC 1990).

Results and discussion

PGPR identification

Isolates, which showed interesting PGPR characteristics, were further identified via sequencing of their 16S rDNA gene. The BLAST searches against the NCBI nucleotide database revealed close relationships to known plant-associated bacteria, including the 23 genera as *Arthrobacter*, *Bacillus*, *Cronobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Lysinibacillus*, *Mesorhizobium*, *Pantoea*,

Pseudomonas, *Staphylococcus*, and *Stenotrophomonas* (Table 3). The most predominant genera of the bacterial isolates were *Pseudomonas* (QUSA-17) (4 species) and *Bacillus* (6 species). These isolates were also found in rhizospheric soil of Himachal Pradesh, India, as reported by Kumar et al. (2012) and used in a biofertilizer formulation. The BLAST search results through NCBI showed the highest (99%) similarity index of isolate QUSA-34 with *Pantoea* sp. The highest sequence resemblance of isolates (QUSA-41 and QUSA-45) with *Pseudomonas* sp. and *Bacillus pumilus* for isolate QUSA-87, as described in Table 2. Hayat et al. (2013) identified 9 Gram-positive bacterial strains from legume rhizospheric soil, using 16S rRNA gene sequencing. These strains showed the highest similarity (97.9–99.8%) to the genus *Bacillus* and were characterized for plant growth promoting (PGP) activities in legume and cereal crops.

Construction of *Pseudomonas* sp. transposon mutants

In this study, transposon mutagenesis of *Pseudomonas* sp. produced mutants that are effective in the protection of alfalfa plants against fungi than the bacterial isolates. Moreover, some mutants of *Pseudomonas* sp. showed more protection of alfalfa plants in soil infected with plant pathogen such as *R. solani* and *Botrytis* sp. than in bacterial isolates. Thus, some genes that mutated are useful target for biocontrol of plants growth promotion agents.

In vitro biocontrol assay of mutagenesis mutants

After 24 h, fungi had spread over the plate towards the bacterial colonies. A clear inhibition zone was observed around mutants 22 and 24 of *P. fluorescens* (isolate 52)

Table 7 Thirty days after inoculation: shoot and root measurements of alfalfa plants under different treatments

No.	Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (mg)	Dry weight (mg)
1	Isolate 52	10.3 c*	5.0 cd	0.8 ef	0.5 ef
2	Mutant 52-12	16.3 a	8.3 a	1.6 a	0.9 a
3	Mutant 52-22	9.0 c	4.3 d	0.9 e	0.5 ef
4	Isolate 45	14.3 ab	7.7 a	1.3 cd	0.7 cd
5	Mutant 45-19	16.7 a	8.0 a	1.4b c	0.7 bc
6	Mutant 45-20	16.0 a	8.0 a	1.3 d	0.6 ed
7	Rh-52	12.0 bc	5.3 bcd	0.7 g	0.4f g
8	Rh-52-12	17.0 a	8.3 a	1.5 ab	0.8 a
9	Rh-52-22	14.3 ab	6.0 abcd	1.5 b	0.7 fcd
10	Rh-45	17.3 a	8.3 a	1.6 d	0.9 ed
11	Rh-45-19	16.0 a	7.7 a	1.5 b	0.8 ab
12	Rh-45-20	15.3 ab	6.7 abc	0.8 f	0.4 fg
13	Rh	0.0 d	0.0 e	0.0 h	0.0 fh
14	Seeds	10.7 c	6.0 abcd	0.8 f	0.3 g

Seeds (Control 1) not inoculated with any microbe; Rh (Control 2) only inoculated with *Rhizoctonia solani*

*Significant at the 0.01 probability level

compared with wild type and the other transposon mutants. An absence of mycelium was observed in the inhibition zone around the colonies (Table 4). Moreover, there was no difference between the wild type and mutant 20 of *P. fluorescens* (isolate 45) in fungi inhibition zone (Table 5). This suggested that some mutant's genes played an important role in inhibiting pathogen growth in vitro.

It was previously reported that the plant growth promotion and suppression pathogenic disease caused by *Pythium* using *Pseudomonas* strains such as SBW25 and CHAO (Naseby et al. 2001). This study found that *P. fluorescens* and some mutants (isolate 52-mutant #22 and isolate 45-mutant #20) had the ability to inhibit the growth of two plant pathogens (*R. solani* and *Botrytis* sp.). It was found that the fungal and oomycete growth was also inhibited by *P. fluorescens* and some mutants (isolate 52-mutant #22 and isolate 45-mutant #20), but that was not found with the viscosin mutants and thus corroborate with the observation of De Bruijn et al. (2007), i.e., the production of viscosin biosurfactants caused zoospore lysis in the oomycete plant pathogen *Phytophthora infestans*.

In vivo antagonism of isolates (37, 45, and 52) and its mutants against *R. solani* and *Botrytis* sp.

Shoot and root lengths, and the fresh and dry weight tendency of surviving plants were significantly affected by inoculated bacteria in alfalfa plants grown in the experiments (Tables 6 and 7). The shoot and root lengths of alfalfa plants (Table 6) grown in the soil treated with the isolate *Bacillus* sp. were significantly higher than (20.7 cm and 8.7 cm, respectively) with the other treatments. Dissimilarity was observed in fresh and dry weight in soil treated with *P. fluorescens* against *Botrytis* sp. Isolates 52 and 45 of *P. fluorescens* along with mutants' 52-M12, 45-M19, and 45-M20 observed with a maximum dry weight and length of shoots and roots of alfalfa plants (Table 7). Furthermore, the efficiency of these bacterial isolates and their mutants against *R. solani* was significantly higher than with the control treatment due to an increase in the dry weight of alfalfa under the infection with *R. solani*. Compared with the application of selected bacterial isolates, obtained findings indicate that application of biocontrol agent could significantly boost the capability to suppress soil-born fungi in alfalfa plants. Additionally, *R. solani* and *Botrytis* sp. also significantly reduced the seedling emergence in this study. The effect of *Botrytis* sp. was partially suppressed by *Bacillus* sp. (isolate #37). However, isolate 45 and mutants (M 45-19 and M45-20) of *P. fluorescens* were found to be less protective against *Botrytis* sp. Contrarily, isolates 52 and 45 and mutants (52-M12 and 45-M19) of *P. fluorescens* were more effective against *R.*

solani. While, in root and shoot lengths and weights of alfalfa seedlings, *P. fluorescens* was found most effective against the two pathogens (*R. solani* and *Botrytis* sp.). Seeds treated with the mutants (52-M12 and 45-M19) under infection resulted in shoot and root length and weight as seen with the mutants (52-M12 and 45-M19) without infection of *R. solani*. Therefore, this disease might be suppressed by plant growth promoting bacteria (*Pseudomonas* sp.) by reducing the pathogen populations through production of antimicrobials (Naseby et al. 2001). This study proved that some genes in *P. fluorescens* distributed by transposon mutagenesis need to be more investigated to identify the location of these genes and their function.

Conclusion

The results revealed that *P. fluorescens* and mutants (isolate 52-mutant #22 and isolate 45-mutant #20) had the ability to inhibit the growth of two plant pathogens. The bacterial isolates and their mutants against *R. solani* and *Botrytis* sp. were significantly higher than the control. Therefore, the results can suggest that application of biocontrol agents could significantly restrict soil-born fungal pathogens in alfalfa plants.

Abbreviations

BLAST: Basic local alignment search tool; CTAB: Hexadecyltrimethylammonium bromide; ITS: Internal transcribed spacer; LB: Luria broth; OD: Optical density; PCR: Polymerase chain reaction; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PDA: Potato dextrose agar; PGPR: Plant growth-promoting rhizobacteria; QUSA: Qassim University Saudi Arabia; RCBD: Randomized complete block design; rDNA: Ribosomal DNA

Acknowledgements

The author gratefully acknowledges Mr. Taha Abdul Elatif for the molecular analysis. The author thanks Dr. M. I. Motawei for his technical support and help in managing the data. Our appreciation is extended to Prof. H. Mousa for his assistance in reviewing the manuscript and Mrs. Nuriye Peaci for editing the English language in my manuscript.

Authors' contributions

The author performed most of the experimental work and analyzed the data. The author read and approved the final manuscript.

Funding

This project was funded by Qassim University, Kingdom of Saudi Arabia.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on a reasonable request.

Ethics approval

This study was approved by College of Agriculture and Veterinary Medicine, Ethics Committee, Qassim University, Saudi Arabia.

Consent for publication

The author participated in this research article.

Competing interests

The author declares that there are no competing interests.

Received: 4 December 2019 Accepted: 18 February 2020

Published online: 28 February 2020

References

- Alsohim AS (2015) Molecular and physiological characterization of plant growth promoting rhizobacteria from rhizosphere soil in Al-Qassim, Saudi Arabia. *J Food Agric Environ* 13(2):118–121
- Alsohim AS, Al-Mohamed R, Motawei MI (2018) Isolation, screening and characterization of new PGPR isolates from rhizosphere of alfalfa plants. *J Food Agric Environ* 16(2):81–85
- Alsohim AS, Taylor TB, Barrett GA, Gallie J, Zhang XX, Altamirano-Junqueira AE, Johnson LJ, Rainey PB, Jackson RW (2014) The biosurfactant viscosin produced by *Pseudomonas fluorescens SBW25* mediates in vitro spreading motility and plant growth promotion. *Environ Microbiol* 16(7):2267–2281
- Bhattacharjee S, van Ooij C, Balu B, Adams JH, Haldar K (2008) Maurer's clefts of *Plasmodium falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. *Blood* 111:2418–2426
- Busse HJ, Denner EBM, Lubitz W (1996) Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. *J Biotechnol* 47:3–38
- Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC (2006) Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Appl Soil Ecol* 34:33–41
- Chin-A-Woeng TFC, De Priester W, Van Der Bij AJ, Lugtenberg BJJ (1997) Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. *Mol Plant Microbe Interact* 10:79–86
- De Bruijn I, De Kock MJD, Yang M, De Waard P, Van Beek TA, Raaijmakers JM (2007) Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species. *Mol Microbiol* 63: 417–428
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13–15
- Hanna AL, Youssef HH, Amer WM, Monib M, Fayez M, Hegazi NA (2012) Diversity of bacteria nesting the plant cover of north Sinai deserts, Egypt. *J Adv Res.* <https://doi.org/10.1016/j.jare.2011.11.003>
- Hayat R, Ahmed I, Peak J, Ehsan M, Iqbal M, Chang YH (2013) A moderately boron tolerant candidates novel soil bacterium *Lysinibacillus pakistanensis* sp. nov. cand., isolated from soybean (*Glycine max* L) rhizosphere. *Pak J Bot* 45: 41–50
- Janda JM, Abbott SL (2007) 16S rRNA Gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 45:2761–2764
- Karagöz K, Ates F, Karagöz H, Kotan R, Çakmakç R (2012) Characterization of plant growth-promoting traits of bacteria isolated from the rhizosphere of grapevine grown in alkaline and acidic soils. *Eur J Soil Biol*:144–150
- Koskey G, Mburu SW, Kimiti JM, Ombori O, Maingi JM, Njeru EM (2018) Genetic characterization and diversity of rhizobium isolated from root nodules of mid-altitude climbing bean (*Phaseolus vulgaris* L.) varieties. *Front Microbiol* 15(9):968. <https://doi.org/10.3389/fmicb.2018.00968>
- Kumar A, Kumar A, Devi S, Pati S, Paya C, Negi S (2012) Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study recent research in science and technology. 4(1):01–05
- Lin DX, Man CX, Wang ET, Chen WX (2007) Diverse rhizobia that modulate two species of *Kummerowia* in China. *Arch Microbiol* 188:495–507
- Lugtenberg BJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* 39: 461–490
- Mignard S, Flandrois JP (2006) 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. *J Microbiol Methods* 67:574–581
- MSTATC (1990) A microcomputer program for the design, management and analysis of agronomic research experiments. Michigan State University, East Lansing
- Naseby DC, Way JA, Bainton NJ, Lynch JM (2001) Biocontrol of *Pythium* in the pea rhizosphere by antifungal metabolite producing and non-producing *Pseudomonas* strains. *J Appl Microbiol* 90:421–429
- Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. *FEMS Microbiol Rev* 25:39–67

Simons M, Van Der Bij AJ, Brand I, De Weger LA, Wijffelman CA, Lugtenberg BJJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* 9:600–607

Vessey JK, Cheminigwa GN (2006) The genetic diversity of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. *Soil Biol Biochem* 38:153–163

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen® journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► [springeropen.com](https://www.springeropen.com)