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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Components of rhizospheric bacterial communities of barley and their potential for plant growth promotion and biocontrol of *Fusarium* wilt of watermelon

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Abstract

This work aimed to characterize antagonistic bacteria from the field-grown barley rhizosphere, and evaluate their potential for growth promotion and biocontrol of *Fusarium* wilt on watermelon caused by *Fusarium oxysporum* f. sp. *Niveum* (FON). Seven bacteria were isolated and screened for plant growth promoting and antagonistic traits. Based on the results of phenotypic characterization and 16S rRNA gene sequencing, the isolates were identified to be related to *Bacillus methylotrophicus* (DMK-1), *Bacillus amyloliquefaciens subsp. plantarum* (DMK-7-2), *Bacillus cereus* (DMK-12), *Pseudomonas brassicacearum* subsp. *brassicacearum* (DMK-2), *Pseudomonas veronii* (DMK-3), *Paenibacillus polymyxa* (DMK-8), and *Ensifer adhaerens* (DMK-17). All the isolates were positive for the production of indole-3-acetic acid (IAA) and ammonia (NH_3), while negative for the production of hydrogen cyanide (HCN). Six bacteria strains (except DMK-17) were able to phosphate solubilization. All the bacteria strains, except DMK-8, were able to produce iron siderophore complexes, and possessed the proteolytic activity. Greenhouse experiment indicated six strains can decrease diseased percentage caused by FON. All the isolates enhanced plant biomass, six strains increased root volume, six strains increased root system activity in greenhouse test. Inoculation of mixtures of seven plant growth promoting rhizobacteria could be more effective in plant growth promotion and biocontrol of *Fusarium* wilt in watermelon.

Keywords Barley · Biocontrol · *Fusarium* wilt · Growth promotion · Rhizosphere bacteria

Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) is one of the worldwide economically important crops. In long-term continuous monocropping field, watermelon plant suffered from serious disease and growth inhibition caused by continuous monocropping obstacle. The syndrome of continuous monocropping obstacle involves soil secondary salinization, reduced diversity of soil microbial communities, and accelerated accumulation of soilborne pathogens [1–3]. Continuous monocropping obstacle results in dramatic decline of yields and quality of watermelon, being a major limiting factor in watermelon production.

Intercropping is a sustainable farming practice that has been widely applied in agroecosystems of China for thousands of years [4]. Various intercropping practice has been applied to alleviate continuous monocropping obstacle or suppress crop disease [5, 6]. However, the efficacy of intercropping to relieve continuous monocropping obstacle is unstable, because many factors, for example, species, cultivars, growth season [7], and/or soil phosphorus availability [8] influence the effectiveness of intercropping. Barley-watermelon relay intercropping system is an empirical farm practice applied in China for hundreds of years. This intercropping system can improve watermelon growth and yield and alleviate *Fusarium* wilt of watermelon. However, the mechanisms of this intercropping system on disease suppression and plant growth promotion has not been fully clarified so far. Inhibition of soil-borne pathogens by intercrop species is a mechanism of intercropping advantage [9]. It is well known that plant growth

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promoting rhizobacteria (PGPR) benefit plants by suppressing disease, stimulating growth, and inducing systemic resistance [10]. Thus, we hypothesized the PGPRs from barley that benefit watermelon plants accumulated in barley-watermelon intercropping system. The PGPRs endowed watermelon plant with growth promotion and disease suppression potential.

Numerous studies on the isolation, screening, and utilization of PGPR are available. Some PGPR strains from genus *Agrobacterium*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Delfitia*, *Exiguobacterium*, *Methylobacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, and *Serratia* have been successfully commercialized [11, 12]. Among the PGPRs, strains from *Bacillus* and *Pseudomonas* are most studied and exploited. In addition, some strains from *Paenibacillus* and *Ensifer* were reported to be potential candidates for biocontrol of plant disease [11–13].

It is important to evaluate the potential of indigenous bacterial populations associated with barley rhizosphere for growth promotion in watermelon. Hence, we focused on the beneficially microbial strains from barley rhizosphere with plant growth promoting activity and antagonistic activity against *Fusarium oxysporum* f. sp. *Niveum* (FON). The main objectives for this work were (1) to isolate and identify promising PGPR from rhizosphere of barley, (2) to screen these PGPR in vitro antagonistic and plant growth promoting activities, and (3) to evaluate the in vivo biocontrol potential against the Fusarium wilt caused by FON and their growth-promoting effects on watermelon plants.

Materials and methods

Isolation of antagonistic bacteria

Antagonistic bacteria were isolated from the field-grown barley rhizosphere. The barleys were planted in the experimental field of Huazhong Agricultural University, Wuhan, China. 0.5 g of root sample was shaken in 100 mL sterilized deionized water for 20 min. The soil suspension was then serially diluted and spread on Luria-Bertani (LB) plates. After incubating at 30 °C for 48 h, single bacterial colonies were selected and streaked onto a new nutrient agar (NA, peptone 10.0 g/L, beef extract 10.0 g/L, sodium chloride 5.0 g/L, agar 12.0 g/L, pH after sterilization 7.3) medium plate. The purified colonies were preserved in LB liquid medium containing 10% glycerol at − 80 °C.

Antagonistic activity against FON of the isolates was evaluated on potato dextrose agar (PDA) plates by dual culture technique. Bacterial isolates were incubated in LB plates at 25 °C. Fungal pathogen was grown on PDA plates. Five-day-old mycelial disc (5 mm) was placed in the center of 9-cm Petri dish PDA plates. An exponentially growing bacterial culture (10^8 CFU/mL) was spotted 3 cm juxtaposed from the fungal disc. Dual cultures were incubated at 28 °C for 7 days, and the diameter of fungal mycelial growth was measured using a ruler, and compared to the control (without any bacterial isolate). The percentage of inhibition was calculated as: % inhibition = [1 − (fungal growth /control growth)] × 100. This experiment was replicated three times.

Identification of the selected bacteria

The DNA of the antagonistic bacteria was extracted and purified with a commercial DNA extraction kit (TransGen Biotech, China), according to the manufacturer's instruction. The extracted DNA was amplified using primers B27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and U1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The PCR mixture contained 2.0 μL of 10 × Taq buffer, 1.6 mL of $MgCl_2$ (25 mM), 1.6 mL of dNTP (2.5 mM), 1.0 mL of each primer, 0.5 mL of DNA template, 0.2 mL of Taq DNA polymerase (10,000 U mL^{-1}), and water to 20 μL. The thermocycling conditions were 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 51 °C, and 1 min 30s at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were then sequenced using an automated DNA sequencer (ABI PRISMTM 3730XL DNA Analyzer). The resulting sequences were subjected to Blast search in NCBI Nucleotide Sequence Database. The unrooted tree was built by the Neighbor Joining with Jukes-Cantor method using Clustal X version 2.0.11 and MEGA version X. Bootstrap replication (1000 replications) was used as a statistical support for the nodes in the phylogenetic trees.

Production of antibacterial metabolites of the antagonistic bacteria

The production of siderophore was determined by Chrome Azurol S (CAS) assay [14]; the protease activity was screened using skim milk agar medium [15]; the production of hydrogen cyanide (HCN) was determined by the picrate assay [16]; the production of ammonia was tested by the method described by Cappuccino and Sherman (1992) [17]; phosphate solubilization ability was tested via NARIP agar plate assay [18]. Quantitative estimation of IAA production was carried out by the Salkowski reagent [19]. Pure IAA (Sigma-Aldrich Co.) was used to prepare standard concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 mg L^{-1}.

Antagonistic effect and plant growth promotion bioassays in vivo

Bacterial strains were cultured in 250-mL conical flasks containing 150 mL LB broth on an orbital shaker at 180 rpm and 30 °C for 24 h. Cells were harvested at stationary phase by centrifugation at 5000×*g* for 10 min, washed twice then resuspended in a sterile phosphate buffer (100 mM, pH 7.0). The harvested bacterial suspension was adjusted to 10^8 CFU/mL and stored at 4 °C before use.

The FON strain was cultured in PDA liquid medium at 180 rpm and 30 °C for 72 h. The cultures were centrifuged at 5000×*g* for 15 min, washed, and resuspended in sterile water. Ten microliters of resuspended spore suspension was loaded in both chambers of a hemacytometer under a coverslip and examined with a microscope at × 400. Spore numbers in five squares (each square contained 16 smaller squares) were counted in each chamber, and counted on both sides were averaged (N). The number of spores per mL (4×10^8 CFU/mL) was calculated by the following equation: spore concentration = $N/80 \times 400 \times 10^4$.

Greenhouse experiments were conducted to examine the efficacy of antagonistic bacterial strains for *Fusarium* wilt control and growth promotion effects on watermelon. Watermelon (cv. Zaojia 8424) seeds were surface sterilized with 1.5% sodium hypochlorite for 10 min and thoroughly washed with sterile deionized water for five times. Seeds were placed in sterile Petri dishes containing moist filter paper at 30 °C in the dark. Two germinated seeds were transplanted into an 8-cm-diameter pot containing 500 g soil. The soil used for the pot experiment was collected from greenhouse of Huazhong Agricultural University, Wuhan, China. The soils were steam sterilized at 121 °C for 1 h successively for three times.

The treatments with only soil (without PGPR) used as control. Two sets of treatments combinations were made. The first set was for testing growth promotion, the second for testing the biocontrol potential of selected PGPR against *Fusarium* wilt of watermelon. For the biocontrol assay, the soils were inoculated with spore of FON to a final density of 10^6 CFU/g dry weight soil 5 days before seedling transplantation. Seven days after transplantation, the cell suspension of the isolates were inoculated into the pot soil with final density of approximately 10^8 CFU/g dry weight soil. In co-inoculation experiment, the cell suspensions of seven strains were mixed in a ratio 1:1 and vortexed.

Pot experiments were carried out in a completely randomized design in a greenhouse (temperature 23–33 °C and relative humidity 65–85%) with 15 pots (each pot contained two plants), thereby, making a total of 30 plants per treatment. The plants were harvested after 45 days of the antagonistic bacteria inoculated treatment, and divided into shoot, root, and leave for analysis of different growth parameters. The root system activity was assayed by a modified triphenyltetrazolium chloride (TTC) test procedure [20]. Each treatment included three replications with six plants per replication.

Statistical analysis

All results presented were the means and standard error of three replicates (means ± SE). The statistical calculations were analyzed by one-way analysis of variance (ANOVA), and compared at 5% level of significance. SPPS Base 10 for Windows (SPSS, Inc., Chicago, IL) was used for all data analyses.

Results

Isolation and identification of antagonistic bacteria strains

The results from the dual culture tests, which were used to evaluate the antagonistic activity against FON of the isolates, were shown in Fig. 1. Seven isolates exhibited different antagonistic activity against FON (Table 1).

Morphological analysis results were presented in Table 2. All the isolates were assessed by comparing 16S rDNA sequences with the GenBank database and reference strains. The generated phylogenetic tree, using phylogenetic analysis of 16S rDNA sequences with existing sequences in GenBank database and reference strains, was presented in Fig. 2. Maximum identities for each isolate were between 95 and 100% with E-value of 0. The distributions were genetically diverse on species of *Bacillus* sp., such as *B. amyloliquefaciens*, *B. methylotrophicus* and *B. cereus*, *Pseudomonas sp.*, such as *P. veronii* and *P. brassicacearum*, *and Paenibacillus polymyxa*, *and Ensifer adhaerens* (Table 3).

Plant growth promoting traits of the antagonistic bacteria strains

The IAA produced by the seven strains were quantitatively determined, as shown in Table 4. All the bacteria strains were able to produce IAA in broth supplemented with and without L-tryptophan. In the broth supplemented with L-tryptophan, DMK-8 produced the highest value of IAA (14.73 ± 0.30 mg L^{-1}), while DMK-3 produced the lowest value of IAA (8.07 ± 0.54 mg L^{-1}). In the broth without L-tryptophan, DMK-8 produced the highest value of IAA (9.26 ± 0.04 mg L^{-1}), while DMK-12 produced the lowest value of IAA (3.80 ± 0.03 mg L^{-1}).

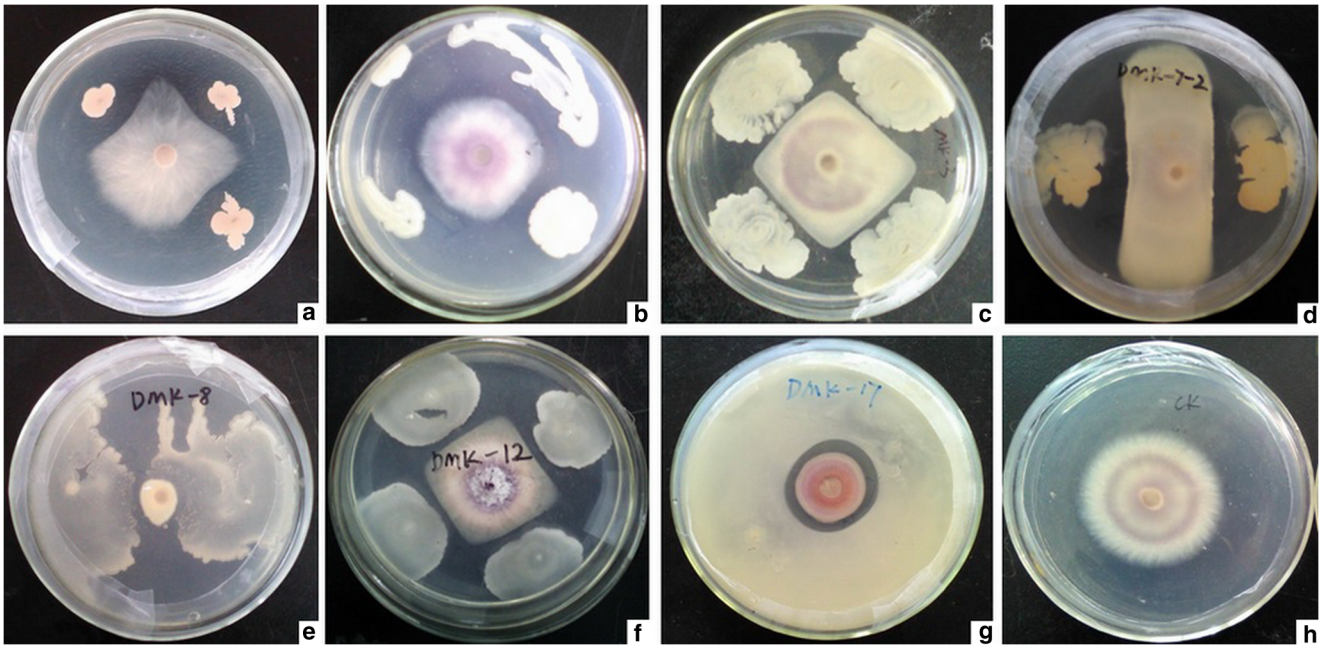

Fig. 1 Photographic indices showing the antifungal potentials of selected antagonistic bacteria strains against *Fusarium oxysporum* f. sp. *niveum* (FON). **a** DMK-1. **b** DMK-2. **c** DMK-3. **d** DMK-7-2. **e** DMK-8. **f** DMK-12. **g** DMK-17. (**h**) CK FON

All bacteria strains, except DMK-17, were tricalcium phosphate solubilizers, as tested via NARIP agar plate assay to produce a transparent halo. All the bacteria strains, except DMK-8, were able to produce iron siderophore complexes in CAS-blue agar with a color change from blue to yellow (or orange) in the medium. None of the bacteria strains were detected positive for HCN production. All the bacteria strains, except DMK-8, showed proteolytic activity, as tested via skim milk medium plate assay to produce a transparent halo. All the bacteria strains were able to produce NH_3 with a color change in the liquid medium after Nessler's reagent adding.

Protection effect of the antagonistic bacteria against *Fusarium* wilt of watermelon

In the pot experiment under greenhouse conditions, 50% diseased watermelon plants were observed in non-antagonistic bacterium inoculated treatment. Co-inoculation with the seven strains significantly decreased diseased percentage of watermelon. When inoculated with single strains, six strains (all except DMK-2) significantly decreased diseased percentage of watermelon (Fig. 3).

Table 1 In vitro growth inhibition of *Fusarium oxysporum* f. sp. *Niveum* (FON) by bacterial isolates obtained from barley rhizosphere

Isolates	Growth inhibition (%)
DMK-1	0.19 ± 0.09
DMK-2	0.09 ± 0.03
DMK-3	0.30 ± 0.14
DMK-7-2	0.63 ± 0.22
DMK-8	0.50 ± 0.13
DMK-12	0.33 ± 0.12
DMK-17	0.60 ± 0.15

Growth promotion effect of the antagonistic bacteria on watermelon plants

Compared to non-inoculated plants, co-inoculated with seven antagonistic bacteria significantly enhanced the fresh weight and dry weight, root volume, and root system activity of watermelon plants (Table 5). For single-isolate inoculation, all the isolates significantly increased plant biomass (both fresh weight and dry weight); all strains, except DMK-3, significantly increased root volume; all strain, except DMK-12, significantly increased root system activity compared to uninoculated plant (Table 5).

Discussion

In the present study, seven strains with antagonistic activity against FON were isolated from rhizosphere of barley plants. Evidence of in vitro and in vivo tests suggested that the isolates benefit watermelon plants via multiple modes of action including antibiosis against FON and plant growth promotion.

Strains from *Bacillus*, including *B. amyloliquefaciens*, *B. methylotrophicus*, and *B. cereus*, were well-documented to be able to successfully colonize the roots and rhizosphere

Table 2 Morphological characteristics of bacterial isolates obtained from barley rhizosphere

Strains	Colony morphology	Gram stain
DMK-1	Thin flat, white color, opaque, round, irregular edge	+
DMK-2	Thin flat, white color, opaque, Round, smooth edge	–
DMK-3	Thin flat, white color, transparent, smooth edge	–
DMK-7-2	Thin flat, white, opaque, round, smooth edge	+
DMK-8	Flat, creamy white, opaque, round, irregular edge	+
DMK-12	Flat, gray white color, round, opaque, smooth edge	+
DMK-17	Thin flat, white, round, opaque, smooth edge	–

of several crops, vegetables, fruit trees, and medicinal herbs, and result in plant growth promotion and disease resistance [21–26]. In the present study, three strains from *B. amyloliquefaciens*, *B. methylotrophicus*, and *B. cereus*, respectively, were isolated from barley rhizosphere. All the three isolates exhibited an antifungal activity against FON in vitro, while the antifungal efficiency among the strains was different. *B. amyloliquefaciens* showed greater antagonistic activity against FON in vitro, but it was not successful as much as *B. methylotrophicus* in greenhouse test.

Strains from *Pseudomonas* strains are also intensively studied PGPR with great biocontrol activity and plant growth

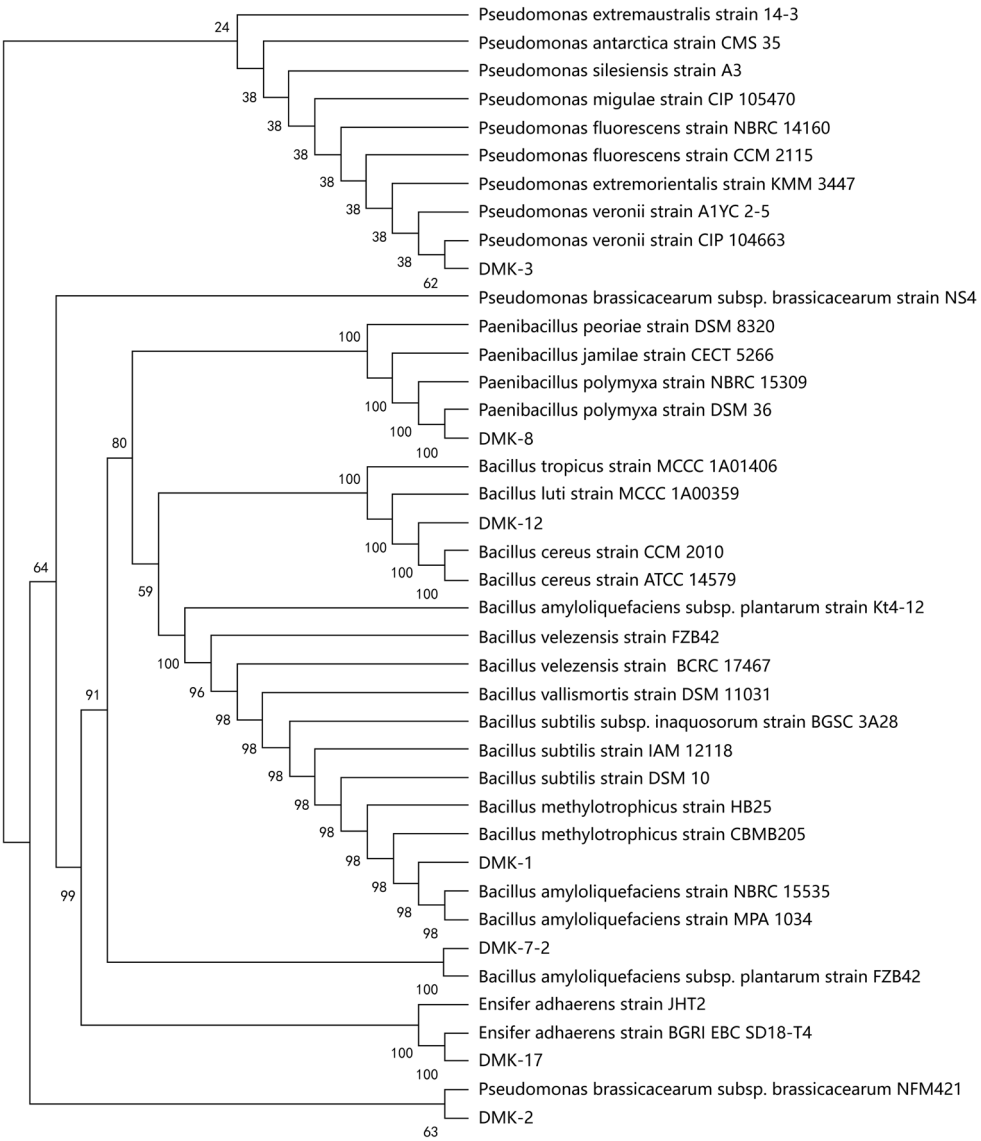

Fig. 2 Neighbor-joining trees showing the position of the seven antagonistic bacteria strains isolated from barley rhizosphere among the related taxa based on 16SrRNA gene sequences. The numbers at branch points were the significant bootstrap values (expressed as percentages based on 1000 replicates). The horizontal branch lines are proportional and indicate the *p*-distances

Table 3 Identification of bacterial isolates obtained from barley rhizosphere by 16S rRNA gene sequence analysis

Isolate	Closest match in NCBI database (accession number)	E value	Identity (%)
DMK-1	*Bacillus methylotrophicus* (HB25)	0.0	99%
DMK-2	*Pseudomonas brassicacearum* subsp. *brassicacearum* (NFM421)	0.0	99%
DMK-3	*Pseudomonas veronii* (CIP 104663)	0.0	99%
DMK-7-2	*Bacillus amyloliquefaciens subsp. plantarum* (FZB42)	0.0	99%
DMK-8	*Paenibacillus polymyxa* (DSM36)	0.0	99%
DMK-12	*Bacillus cereus* (ATCC 14579)	0.0	99%
DMK-17	*Ensifer adhaerens* (NBRC 100388)	0.0	99%

promoting effects. Species from *P. veronii* and *P. brassicacearum* are root-associated strain, having a wide spectrum of antagonistic activity against plant pathogens and strong plant growth-promoting effects [27–29]. In this study, two *Pseudomonas* strains were isolated from barley rhizosphere, i.e., DMK-2, DMK-3. Both strains exhibited great antifungal activity against FON in vitro as well as in the greenhouse test.

P. polymyxa has been proved to be an agriculturally important microbe for its great plant growth-promoting abilities, broad spectrum of antagonistic activity against plant pathogens, and wide range of host plant [12]. Many strains from *P. polymyxa* were considered to be promising biological control agent [12]. Specially, *P. polymyxa* E681 has been successfully applied in biofertilizer to control the *Fusarium* wilt caused by FON for its great disease suppression capability [30–32]. In this study, strain DMK-8 isolated from barley rhizosphere exhibited great antifungal activity both in vitro and in greenhouse test, in line with these results.

Strain from *Ensifer adhaerens* (strain DMK-17) showed the antifungal activity both in vitro and in the greenhouse test in the present study. Similarly, Fan et al. reported that strain of *Ensifer adhaerens* benefited plant by suppressing several plant diseases and promoting plant growth [13].

In general, the direct mechanism of plant growth promotion by PGPR is providing the plant with compounds by which stimulating growth and development, or facilitating uptake of certain nutrients [33]. The capacity of IAA production, NH_3 production, HCN production, siderophore production, and phosphate-solubilization ability have been intensively studied. In this present study, all the selected strains showed in vitro IAA production ability. Patten and Glick reported that 80% of microorganisms isolated from the plant rhizosphere can synthesize and release auxins [34]. IAA is the phytohormone known to stimulate root growth and development and facilitate uptake of certain nutrients [34]. Regarding phosphate solubilization, strain DMK-17 in the present study lacked the ability of solubilize inorganic phosphate. Bacteria belonging to genus *Bacillus* and *Pseudomonas* are well-known significant phosphate solubilizing bacteria [10]. Results from the present study are in line with these conclusions. All strains, except DMK-8 in the current study, were confirmed to produce siderophores by CAS-blue agar assay. All strain can produce ammonia from nitrogen containing organic matters. Some *Bacilli* and *Pseudomonas* species, for example *B. methylotrophicus* strain CKAM and *Pseudomonas fluoroscens* strain showed HCN production capacity [35]. However, none of the strains in this study exhibited the HCN production capacity. All strains, except DMK-2 and DMK-8, have the proteolytic activity. Therefore, a variety of mechanisms, including production of growth-promoting substance, solubilization of

Table 4 Different plant growth-promoting traits of selected antagonistic bacterial isolates

Bacterial isolates	IAA production		Siderophore production	HCN production	Phosphate solubilization	Proteolytic activity	NH_3 production
	With L-Try	Without L-Try					
DMK-1	11.33	6.23	+	–	+	+	+
DMK-2	11.53	7.14	+	–	+	–	+
DMK-3	10.75	6.25	+	–	+	+	+
DMK-7-2	8.07	5.58	+	–	+	+	+
DMK-8	14.73	9.26	–	–	+	–	+
DMK-12	8.63	3.80	+	–	+	+	+
DMK-17	13.02	4.32	+	–	–	+	+

(+) = positive production; (−) = negative production

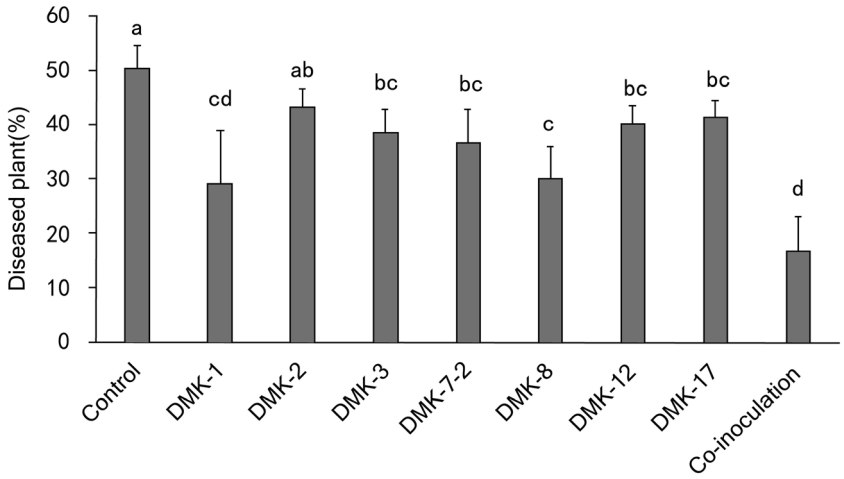

Fig. 3 Effect of antagonistic bacteria inoculation on disease incidences of watermelon plant. Results are means ± SE, and different letters indicated statistically significant differences between treatments, $p < 0.05$

minerals such as P, and production of functional enzymes may contribute to growth promotion and biocontrol activities of the isolated strains in the present study.

PGPR decrease or prevent the deleterious effects of certain phytopathogen by altering the composition and function of the rhizosphere microbial community [33]. In the present study, inoculation with some PGPR significantly decreased diseased plant and increased plant biomass of watermelon, and co-inoculation of the mixtures of PGPR achieved more effective disease suppression and plant growth promotion in the greenhouse test. Mixtures of PGPR strains showed more effective biocontrol, and plant growth promotion activity has been confirmed due to synergistic modes of direct and indirect action of PGPR [36–38]. The development of soil-borne diseases *Fusarium* wilt in watermelon caused by FON is due to a decline of the soil microbial diversity and alteration in the rhizosphere microbial community [39, 40]. Co-inoculation of several strains leads to the alteration of the whole microbial community in rhizosphere niche and results in relieve of symptoms of watermelon continuous monocropping obstacle. Xiong et al. suggested that bio-fertilizer application induces soil suppressiveness against *Fusarium* wilt disease were due to bio-fertilizer reshaping the soil microbiome [41]. Ren et al. reported intercropping with aerobic rice alleviated *Fusarium* wilt in watermelon, by restraining the spore production of *Fusarium* as well as changing the microbial communities in rhizosphere soil [5]. Thus, results in the present study implied PGPR-mediated plant growth promotion contributes to the mechanisms of barley-watermelon relay intercropping system relieving continuous monocropping obstacle of watermelon. Further studies under field conditions and at multiple locations are needed to corroborate the findings of this study. In addition, the isolated strains of the present study can be used together to alleviate continuous monocropping obstacle or control *Fusarium* wilt disease of watermelon after the consistency of PGPR treatments be tested and evaluated in field conditions.

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Table 5 Fresh weight, dry weight, root volume, and root system activity of watermelon plants with and without antagonistic bacteria inoculation. Results are means ± SE, and statistically significant differences between treatments and control are indicated as *, $p < 0.05$

Treatment	Fresh weight (g)	Dry weight (g)	Root volume (cm^3)	Root system activity (μg g^{-1} FW)
Control 1	12.08 ± 0.58	1.31 ± 0.04	4.86 ± 0.14	20.83 ± 0.10
DMK-1	17.13 ± 0.65*	1.80 ± 0.06*	6.83 ± 0.18*	24.59 ± 0.64*
DMK-2	19.55 ± 1.07*	2.05 ± 0.05*	7.14 ± 0.18*	26.63 ± 1.33*
DMK-3	15.70 ± 0.54*	1.76 ± 0.05*	5.14 ± 0.30	23.69 ± 1.08
DMK-7-2	14.25 ± 0.68*	1.54 ± 0.07*	7.12 ± 0.27*	24.64 ± 0.99*
DMK-8	17.17 ± 0.55*	1.82 ± 0.07*	7.29 ± 0.37*	26.24 ± 1.33*
DMK-12	14.28 ± 0.80*	1.62 ± 0.07*	6.03 ± 0.31*	22.32 ± 1.12
DMK-17	15.35 ± 0.86*	1.61 ± 0.08*	7.32 ± 0.27*	24.05 ± 0.64*
Co-inoculation	25.24 ± 0.75*	2.67 ± 0.06*	11.33 ± 0.28*	40.79 ± 0.99*

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