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Characterization of *Bacillus* Isolates from the Rhizosphere of Tomato Suppressing *Fusarium* Wilt Disease

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Fusarium wilt of tomato is one of the most prevalent and economically important diseases of tomato worldwide especially in tropical regions. The aims of the present study were to isolate and characterize *Bacillus* bacteria from tomato rhizospheric soil of various regions in Iran and determine the isolates that exhibit high levels of antagonistic efficiency against tomato *Fusarium* wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and growth promotion activity. In this study, 303 *Bacillus* isolates were obtained from tomato rhizospheric soil. Dual culture and volatile metabolite tests were used to screen for antagonism of *Bacillus* isolates against *Fol*. Among them, 20 isolates were found to inhibit pathogen growth by 67.77% and 33.33% in dual culture and volatile metabolite tests, respectively. Based on the results of physiological tests and 16S rRNA and *gyrA* gene sequence analysis of 20 effective isolates, 11, seven and two isolates were identified as *B. subtilis*, *B. velezensis* and *B. cereus*, respectively. The results of greenhouse assessment showed that KR1-2, KR2-7 and A2-9 isolates which were characterized as *Bacillus subtilis*, reduced the disease index to 16.67% and promoted the plant growth by 80%. These isolates may serve as potential promising biocontrol agents against *Fusarium* wilt of tomato.

Keywords: *Fusarium* wilt, tomato, *Bacillus*, antagonism, growth promotion, biocontrol agents.

Tomato (*Solanum lycopersicum*) is one of the most consumed vegetables and the seventh most important crop in the world because the worldwide production of tomato reached almost 177 million tons in 2016 (FAOSTAT, http://www.fao.org/faostat/en/#data/QC). The Asian countries dominate global tomato market among which Iran ranks 6th, producing 37 tons per hectare (Bergougnoux, 2014). Many constrains reduce the productivity and quality of tomato, among which diseases, especially fungal diseases play a remarkable role. Tomato *Fusarium* wilt, caused by a soil-born fungal pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), is one of the economically important fungal diseases that results in a massive loss in production of both field and greenhouse tomatoes worldwide (Agrios, 2005). Its occurrence has been reported in 32 countries especially in countries with warm and humid climate such as United States, Australia, Great Britain, Netherlands, Brazil, Mexico, Morocco, Israel, Iraq (Mui-Yun, 2003) and Iran (Amini, 2009).

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This pathogen attacks the host by destroying the root tissue. It colonizes in the plant xylem, blocks the vascular system and eventually prevents water and nutrients from being transferred to the rest parts of plant host. It causes wilting, discoloration, and finally death of the plant. This pathogen lives as saprophytes in the soil up to ten years (Burgess et al., 2008).

The emergence of new pathogenic isolates and rising threat of fungal plant diseases have led to indiscriminated use of synthetic fungicides that aim at suppressing fungal pathogens and achieving the maximum crop production, which causes serious environmental and health problems. According to the report of the International Labor Organization (ILO) in 2017, a large number of agricultural workers die every year as a result of pesticide poisoning. Focusing more attention on finding environmentally-friendly and food-hygienically-safe alternatives to chemical fungicides for controlling plant pathogens is one of the important aims for many disease control programmes worldwide (Cawoy et al., 2011).

Biological control is a promising alternative to agrochemicals. Although numerous bacteria and fungi with considerable antagonism potential against soil-borne pathogens have been identified, few of them have been commercialized as biocontrol products (Meyer and Roberts, 2002). The majority of commercialized products are bacteria-based biopesticides and about half of them are *Bacillus*-based products because this bacterial genus retains several valuable traits (Cawoy et al., 2011). *Bacillus* spp. form dormant spores that are resistant to extreme pH and temperature and thus can be easily formulated and stored (Stein, 2005). These bacteria are compatible with osmotic conditions. Besides, they colonize root surface, promote plant growth (Turner and Backman, 1991; Cawoy et al., 2011) and protect the plant against pathogen attack using a complex modes of action (Raupach and Kloepper, 1998; Shoda, 2000; Romero et al., 2004). Therefore, the development and utilization of biocontrol products based on these bacteria and or their metabolites are considered as alternative or complimentary methods for chemical control of plant diseases.

Many experimental studies have been done to investigate the biocontrol efficiency of *Bacillus* species against *Fusarium* wilt of tomato. The biocontrol effect of four *Bacillus* species including *B. amyloliquefaciens*, *B. cereus, B. pumilus* and *B. subtilis* were examined against *Fol*. The results of *in vivo* experiments revealed that the tomato plants treated with *B. cereus* represented the least disease incidence (18.75%) and highest percent disease control (81.2%) (Ajilogba et al., 2013). Treated tomato seeds with *B. amyloliquefaciens* (FZB24) resulted in induced-defense enzyme activities and reduced disease incidence (Elanchizhiyan et al., 2018).

Apart from the biocontrol efficiency of *Bacillus* species, these bacteria also have a plant growth promotion effect (Shafi et al., 2017). The inoculation of spores or cell suspension of *B. subtilis* OTPB1 in tomato seeds significantly increased seedling vigor, shoot and root growth and leaf area of tomato under greenhouse conditions (Chowdappa et al., 2013). Qiao et al. (2017) added the cell suspension of *B. subtilis* PTS-394 to pot soil of tomato and observed an increase of 8.90% and 18.30% in plant height and root weight, respectively, compared with untreated control plants. Spraying both sides of watermelon leaves with cell suspension of *B. amyloliquefaciens* strain 54 significantly increased the shoot length of bacterial-treated plants because of enhancing the content of available nitrogen (N), phosphorus (P), potassium (K) in soil and leaf chlorophyll (Jiang et al., 2015).

In the present study, *Bacillus* bacteria were isolated from tomato rhizospheric soil of various regions across Iran. They were evaluated based on growth promotion potential and

antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici*, in order to determine the *Bacillus* bacteria that exhibit high levels of antagonistic and growth promotion activities.

Materials and Methods

Fungal pathogen

Fusarium oxysporum f. sp. *lycopersici* (*Fol*) strain Fo-To-S-V-1 was obtained from the Iranian Research Institute of Plant Protection. It was cultivated on potato dextrose agar (PDA) at 27 °C. The fungal pathogen was sub-cultured onto a fresh PDA plate for further tests.

Isolation of Bacilli

Tomato rhizosphere soil samples were collected from fields in Golestan, Alborz and Khorasan-Razavi provinces of Iran. Plants in each field were randomly selected, uprooted and 20 g of rhizosphere soil closely adhering to root of each plant was harvested. All the rhizosphere soil samples from each field were pooled together to make an admixture sample. Each composite soil sample was placed in the sterile plastic bag, maintained on ice, transferred to the laboratory and stored at 4 °C for further experiments. Soil suspensions were obtained by mixing 10 g of each soil sample in 90 mL sterile water for 30 min at 150 rev min^{-1} on a rotary shaker. Each suspension was diluted from 10^{-1} to 10^{-6} and heated in a water bath at 80 °C for 10 minutes. 200 μl of each dilution was plated onto nutrient agar medium (Shanmugam et al., 2011).

After incubating at 27 °C for three days, numerous single colonies appeared and most of them belonged to *Bacillus* genus according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Each *Bacillus* colony was maintained as a pure culture on a nutrient agar (NA, Merck, Germany; 0.3% beef extract, 0.5% peptone and 1.5% agar) plate with periodic transfer to fresh medium. Besides, they were kept for long-term storage at –80°C in lysogeny broth (LB, Merck, Germany) with 20% glycerol (v/v).

In vitro *screen for antagonistic activity*

All of the obtained isolates were evaluated for antagonistic ability against *Fol* using dual culture and volatile metabolites assays.

Dual culture

In the dual culture test, each bacterial isolate was cultured at a distance of 3 cm from a 5 mm diameter disc of *Fol* fresh culture in 9 cm PDA plates. The control plates were inoculated only with the fungal pathogen. Plates were incubated at 27 °C. The fungal growth was daily checked by measuring the diameter of the colony for a period of three days. The percentage of fungal growth inhibition (PFGI) was calculated by using the formula below, where, R_1 is the maximum radius of the growth fungal colony in control plate and R_2 is the radius of the fungal colony that grew in the presence of bacteria (Riungu et al., 2008):

Volatile metabolite test

In the volatile metabolites assay, each bacterial isolate was inoculated at the center of a plate containing nutrient agar and incubated at 27 °C for 24 h. Then, 5 mm disc of the actively growing culture of the fungal pathogen was placed at the center of a plate containing PDA, inverted over the bacterial culture. Two plates were sealed together with Parafilm® M and further incubated at 27 °C. The control sets did not contain the bacteria (Fiddaman and Rossall, 1993). The fungal growth was daily checked by measuring the diameter of the colony for a period of three days. The percentage of fungal growth inhibition was obtained using the formula as described earlier.

Physiological and molecular characterization of Bacilli

Bacillus isolates showing a broad spectrum of antagonistic activity were subjected to further identification according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and sequence analysis of the 16S rRNA and *gyrA* genes.

For physiological characterization, the gram reaction, motility, growth at 45 °C, growth in 7% NaCl, utilization of citrate, anaerobic growth in glucose broth, acid and gas production from carbohydrates and starch hydrolysis characteristic of *Bacillus* isolates were tested following standard procedures (Holt et al., 1994).

For molecular characterization, genomic DNA of *Bacillus* isolates was extracted from 12 h old liquid cultures of bacterial isolates grown in Lysogeny Broth medium and prepared based on the standard protocol for bacterial genomic DNA preparations (Jasra, 2004).

The amplification of 16S rRNA gene was carried out using a universal primer set consisting of 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′), 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′) (Turner et al., 1999). The PCR reaction was performed according to the thermocycler protocol which included an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 46 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Li et al., 2012).

A primer set consisting of p-gyrA-f (5′-CAGTCAGGAAATGCGTACGTCCTT-3′), p-gyrA-r (5′-CAAGGTAATGCTCCAGGCATTGCT-3′) (Roberts et al., 1994) was used to amplify the *gyrA* gene. The PCR reaction was performed according to the thermocycler protocol which included an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Li et al., 2012).

The PCR products were sequenced by the Beijing Genomics Institute (BGI). The 16S rRNA and *gyrA* gene sequences of the studied bacterial isolates were BLASTed against with known sequences in the NCBI GenBank and the phylogenetic tree was constructed using the maximum likelihood method of MEGA 7.0.26 software (Kumar et al., 2016).

Preparing inocula for in vivo *assessment*

The bacterial isolates that strongly inhibited the growth of the fungal pathogen *in vitro*, were grown separately in nutrient broth at 27 °C for 48 h under constant shaking (150 rpm) to obtain a final concentration of 10^8 CFU ml^{-1} (Chen et al., 2013).

In order to prepare the pathogen inoculum, corn seeds were washed and soaked in tap water for 24 h in 2 L Erlenmeyer flasks. After draining off the excess water, the flasks were filled with corn seeds to one-third and autoclaved twice in two consecutive days. Six 5 mm diameter disks from seven-day-old PDA culture of *Fol* were cultured on sterilized corn seeds in each flask (Leslie et al., 2006). The inoculated flasks were maintained at 27 °C for three weeks. In order to prepare the inoculated soil for *in vivo* assessment, 5 g of infected corn seeds were mixed with 1 kg of sterilized mixture consist of garden soil, sand and fertilizer (2:1:1 at w/w/w).

Assessment of bacterial isolates against F. oxysporum *in greenhouse*

Tomato (*Solanum lycopersicum*) cv. Falat seeds were planted in nursery soil in seedling trays and maintained at 30 °C in a greenhouse with relative humidity between 40% to 50% for six weeks. The seedlings were transplanted to pots containing 3 kg of pre-treated soil with infected corn seeds and bacterial suspensions were soil drenched (100 ml/pot). The same concentration of bacterial suspensions was applied 10 days later by soil drenching. The pots in which no bacteria but the pathogen was applied and the pots without any treatment were considered as infected and non-infected control, respectively. Forty-five days after inoculating the pathogen, disease severity was recorded based on a 0–4 scale in which 0 = no wilt symptoms, 1 = wilt symptoms on 1–25% of the leaves, 2 = wilt symptoms on 26–50% of the leaves, 3 = wilt symptoms on 51–75% of the leaves and 4 = wilt symptoms on more than 76% of the leaves (Chen et al., 2013).

The disease index and biocontrol effect were calculated according to the following formula (Chen et al., 2010):

$$\text{Biocontrol efficacy}\,(\%) = \frac{\text{disease index of pathogen control} - \text{disease index of bacteria treatment}}{\text{disease index of pathogen control}} \times 100$$

In vivo experiment was repeated twice.

Evaluation plant growth promotion activity of Bacilli

The bacterial isolates that strongly inhibited the growth of fungal pathogen *in vitro,* were selected for assessing their plant growth promotion activity in presence of pathogen. In doing so, the height, shoot and root dry weight of plants treated with bacterial isolates and the pathogen simultaneously were compared with those of untreated (non-infected) and the pathogen treated (infected) control plants. At the end of the experiment, the tomato plants were cautiously uprooted and washed off the adhering soil under running tap water. The shoots and roots were separated and shoot height was measured. The dry weight of shoot and root were determined after drying shoots and roots in an oven at 70 °C for three days.

Statistical analysis

The variance analysis and comparison of mean test were done using SAS 9.3 software. A completely randomized design and randomized complete block design with three

replications were used for the *in vitro* and *in vivo* experiments, respectively. The significant differences between treatments were evaluated by Fisher's least significant difference (LSD) test and $P \leq 0.05$ was considered as critical differences.

Results

Isolation, screen, and selection of Bacillus *isolates with antagonistic activity*

Totally, 303 *Bacillus* isolates were obtained from tomato rhizospheric soils of three provinces. Out of these, 108 isolates showed antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici* in dual culture test. Among them, 31 isolates inhibited the fungal mycelial growth more than 50% in the dual culture test (Table 1, Fig. 1). Besides, these 31

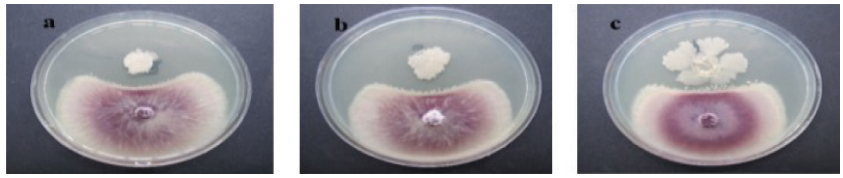

Fig. 1. The growth of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) inhibited by *Bacillus* isolates in dual culture test. Antagonism pattern a) G1-7 b) KR2-7 and c) A2-19 against *Fol*

bacterial isolates also controlled the growth of *Fol* in the volatile metabolite test, but there was variability among them. KR2-7 was the most effective isolate as 46.67% inhibition (Table 1). Based on the results of *in vitro* experiments, 20 *Bacillus* isolates that inhibited the pathogen growth more than 50% and 15% in dual culture and volatile metabolite, respectively, were selected for further study.

Physiological and molecular identification of Bacillus *isolates*

Based on physiological tests, 11 out of 20 antagonistic isolates were characterized as *Bacillus subtilis* and nine remaining isolates, including G1-8, G1-24, G2-21, G2-22, G3-1, G3-3, KR1-23, KR2-5 and A1-34 which belonged to other species of *Bacillus* (Table 2). Results of 16S rRNA and *gyrA* sequence analysis confirmed the identification of *Bacillus subtilis* isolates and G2-22 and A1-34 isolates were identified as *Bacillus cereus*. Moreover, G1-8, G1-24, G2-21, G3-1, G3-3, KR1-23 and KR2-5 were characterized as *Bacillus velezensis*. A phylogenetic tree was constructed based on 16S rRNA and *gyrA* sequences (Fig. 2). The 16S RNA sequences of 20 bacterial isolates were deposited in the NCBI GenBank database.

Table 1

The percentage of *Fusarium oxysporum* f. sp. *lycopersici* growth inhibition by *Bacillus* isolates in dual culture and volatile metabolite tests

The source of bacteria	Isolate code	PFGI* in dual culture test	PFGI* in volatile metabolite test
Golestan province	G1-2	62.22 ± 1.11 abcd	13.34 ± 1.2 gh
	G1-7	61.11 ± 1.2 abcde	13.33 ± 0.69 gh
	G1-8	64.44 ± 2.93 abc	25.56 ± 1.03 c
	G1-11	63.33 ± 5.02 abc	14.29 ± 0.57 fg
	G1-24	63.33 ± 1.92 abc	18.88 ± 1.89 d
	G2-8	63.33 ± 1.9 abc	5.54 ± 0.38 jk
	G2-21	67.77 ± 2.22 ab	18.36 ± 0.85 de
	G2-22	67.77 ± 1.92 ab	15.08 ± 0.62 efg
	G2-29	65.55 ± 1.1 ab	18.76 ± 1.13 d
	G2-38	51.11 ± 2.93 g	17.77 ± 1.11 def
	G2-45	66.67 ± 6.93 ab	10 ± 0.76 hi
	G3-1	67.77 ± 1.2 a	15.06 ± 0.63 efg
	G3-2	65.55 ± 4.83 ab	8.88 ± 0.98 ij
	G3-3	67.77 ± 1.11 ab	23.33 ± 1.53 c
	G3-11	62.22 ± 1.2 abcd	16.66 ± 0 defg
Khorasan Razavi province	KR1-2	61.11 ± 2.93 abcde	26.63 ± 0.87 c
	KR1-23	54.44 ± 2.22 defg	16.66 ± 0.19 defg
	KR2-5	68.88 ± 2.93 a	23.36 ± 1.96 c
	KR2-7	64.44 ± 2.9 abc	46.67 ± 1.92 a
	KR2-8	54.44 ± 4.44 defg	1.1 ± 0.58 l
	KR2-9	52.22 ± 7.28 fg	2.22 ± 0.22 kl
	KR2-15	50.11 ± 1.25 g	32.22 ± 0.97 b
	KR3-10	51.11 ± 2.93 g	31.11 ± 0.8 b
Alborz province	A1-9	51.11 ± 2.8 g	17.77 ± 0.4 def
	A1-34	53.33 ± 2.9 efg	26.67 ± 1.11 c
	A2-4	54.44 ± 3.33 defg	14.44 ± 2.11 fg
	A2-9	52.22 ± 2.76 fg	24.44 ± 1.12 c
	A2-20	52.22 ± 2.83 fg	6.66 ± 1.87 ij
	A2-21	56.67 ± 2.22 cdef	5.55 ± 0.96 jk
	A2-22	54.44 ± 1.92 defg	24.44 ± 0.55 c
	A2-25	53.35 ± 1.39 efg	33.33 ± 2.98 b

Each value represents mean ± standard error from three independent replications. Values with different lowercase letters are significantly different according to LSD test at $P < 0.05$.
*PFGI: The percentage of fungal growth inhibition.

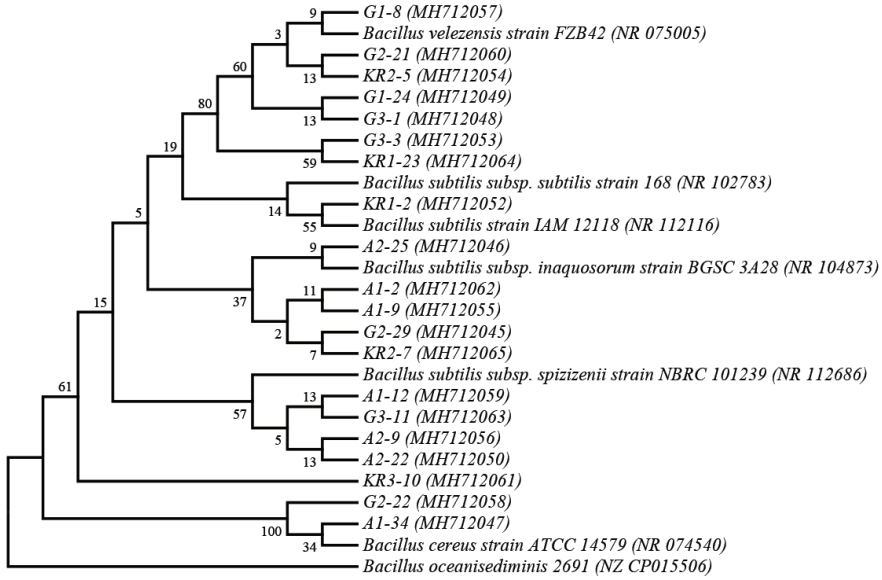

Fig. 2. Phylogenetic trees of 20 *Bacillus* strains based on 16S rRNA and g*yrA* gene sequences. Numbers at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials. The *Bacillus oceanisediminis* (NZ_CP015506) was served as outgroup

Biocontrol of tomato Fusarium *wilt in greenhouse*

The biocontrol efficacy of 20 selected *Bacillus* isolates was evaluated under greenhouse conditions. After eight weeks of inoculation, the initial symptoms of tomato *Fusarium* wilt including yellowing and wilting of the lower leaves appeared and then spread to the upper leaves of plants.

According to the results of *in vivo* biocontrol evaluation (Table 3), the 20 *Bacillus* isolates were categorized into three groups including highly effective, effective, and non-effective isolates. The highly effective group consists of three isolates, KR1-2, KR2-7, and A2-9, as they have shown the lowest disease index percentage (16.67%) without any significant difference from non-infected control and with the highest biocontrol efficiency (80%). Since the effect of G1-8, G2-21, G3-3, G3-11, KR1-35, KR2-5, KR3-10, A1-34 and A2-22 on suppressing the fungal pathogen invasion was less pronounced than that of the more effective group, we therefore categorized these nine isolates as effective biocontrol *Bacillus*. The rest of *Bacillus* isolates, including G1-24, G2-22, G2-29, G3-1, KR1-23, KR2-15, A1-9, A1-12 and A2-25, were considered as non-effective isolates because they did not significantly reduce the disease index percentage in comparison with infected control plants.

Plant growth promotion effect of Bacillus *isolates under greenhouse conditions*

The plant growth promotion potential of 20 *Bacillus* isolates was studied by measuring several growth parameters, i.e. plant height, dry shoot and root weight (Table 4). Twenty isolates were classified into three groups encompassing the highly effective, ef-

Table 2

Differential physiological tests for identifying *Bacillus* isolates

Bacteria stains	Bacillus species (Accession number)	Gram reaction	Motility	Growth at 45 °C	Growth at 65 °C	Growth in 7% NaCl	Utilization of citrate	Anaerobic growth in glucose broth	Acid and gas production from carbohydrate		Starch hydrolysis
									Open tube	Sealed tube	
G1-8	*B. velezensis* (MH712057)	+	+	+	+	+	–	–	yellow	yellow	+
G1-24	*B. velezensis* (MH712049)	+	+	+	+	+	–	–	yellow	yellow	+
G2-21	*B. velezensis* (MH712060)	+	+	+	+	+	–	+	yellow	yellow	+
G2-22	*B. cereus* (MH712058)	+	+	+	+	+	+	+	green	green	+
G2-29	*B. subtilis* (MH712045)	+	+	+	+	+	+	–	yellow	yellow	+
G3-1	*B. velezensis* (MH712048)	+	+	+	+	+	–	–	yellow	yellow	+
G3-3	*B. velezensis* (MH712053)	+	+	+	+	+	–	–	yellow	yellow	+
G3-11	*B. subtilis* (MH712063)	+	+	+	+	+	+	–	yellow	yellow	+
KR1-2	*B. subtilis* (MH712052)	+	+	+	+	+	+	–	yellow	yellow	+
KR1-23	*B. velezensis* (MH712064)	+	+	+	+	+	+	–	yellow	yellow	+
KR2-5	*B. velezensis* (MH712054)	+	+	+	+	+	–	–	yellow	yellow	+
KR2-7	*B. subtilis* (MH712065)	+	+	+	+	+	+	–	yellow	yellow	+
KR2-15	*B. subtilis* (MH712051)	+	+	+	+	+	+	–	yellow	yellow	+
KR3-10	*B. subtilis* (MH712061)	+	+	+	+	+	+	–	yellow	yellow	+
A1-9	*B. subtilis* (MH712055)	+	+	+	+	+	+	–	yellow	yellow	+
A1-12	*B. subtilis* (MH712059)	+	+	+	+	+	+	–	yellow	yellow	+
A1-34	*B. cereus* (MH712047)	+	+	–	+	–	+	+	green	green	+
A2-9	*B. subtilis* (MH712056)	+	+	+	+	+	+	–	yellow	yellow	+
A2-22	*B. subtilis* (MH712050)	+	+	+	+	+	+	–	yellow	yellow	+
A2-25	*B. subtilis* (MH712046)	+	+	+	+	+	+	–	yellow	yellow	+

*V: between 21–70% of strains are positive.

Table 3

The biocontrol effect of 20 selected *Bacillus* isolates on *Fusarium* wilt of tomato

Treatment	Disease index (%)	Biocontrol efficacy (%)
Infected control	77.78 a	–
Non-infected control	16.67 e	–
G1-8	27.78 de	66.67 ab
G1-24	66.67 ab	20 de
G2-21	27.78 de	66.67 ab
G2-22	77.78 a	6.67 e
G2-29	55.56 abc	33.33 cde
G3-1	72.22 a	13.33 e
G3-3	27.78 de	66.67 ab
G3-11	22.22 de	73.33 ab
KR1-2	16.67 e	80 a
KR1-23	61.11 ab	26.67 de
KR2-5	22.22 de	73.33 ab
KR2-7	16.67 e	80 a
KR2-15	55.56 abc	33.33 cde
KR3-10	33.33 cde	60 abc
A1-9	55.56 abc	33.33 cde
A1-12	55.56 abc	33.33 cde
A1-34	44.44 bcd	46.67 bcd
A2-9	16.67	80 a
A2-22	22.22 de	73.33 ab
A2-25	55.56 abc	33.33 cde

Values with different lowercase letters are significantly different according to the LSD test at $P < 0.05$.

fective and non-effective Plant Growth Promoting Rhizobacteria (PGPR). KR1-2, KR2-7 and A2-9 were the highly effective PGPR agents. G2-22, KR1-23, KR2-15, A1-9, A1-12 and A1-34 were categorized as non-effective PGPR agents because the growth parameters of treated plants with these bacterial isolates did not present a significant difference compared to infected control plants. The remaining 8 *Bacillus* isolates especially G3-3, KR2-5, KR3-10 and A2-22 were grouped as effective PGPR agents because the inoculated plants by these *Bacillus* isolates showed growth parameters close to those of non-infected control plants (Table 4).

Discussion

Antagonistic bacteria are widely utilized for biological control of soil-borne phytopathogens to reduce agrochemical consumption, facilitate sustainable agriculture implementation, and promote plant growth. In order to control various fungal phytopathogens within an acceptable level, numerous research attempts using *Bacillus* spp. as biocontrol

Table 4

The influence of *Bacillus* isolates on growth parameters of tomato under greenhouse conditions

PGPR group	Treatment	Plant height (cm)	Dry shoot weight (g)	Dry root weight (g)
–	Infected control	59.33 ± 1.2 k	6.73 ± 0.88 h	4.4 ± 0.24 kl
–	Non-infected control	96.67 ± 2.33 ab	12.36 ± 0.33 a	9.56 ± 0.26 a
Highly effective PGPR	KR1-2	97.33 ± 2.91 a	11.8 ± 0.95 ab	9 ± 0.36 ab
	KR2-7	94 ± 2.08 abc	10.8 ± 0.3 abcd	8.63 ± 0.24 abc
	A2-9	90.33 ± 1.45 abcde	10.16 ± 0.46 abcd	8.56 ± 0.34 abcd
Effective PGPR	G1-8	87.66 ± 1.2 abcdef	9.06 ± 0.4 def	7.26 ± 0.21 efg
	G1-24	79.66 ± 1.2 fghi	9.26 ± 0.08 de	6.03 ± 0.12 ghij
	G2-21	83 ± 2.08 defg	8.67 ± 0.98 efgh	7.23 ± 0.83 efg
	G2-29	73 ± 8.19 ghij	9.13 ± 0.24 def	6.06 ± 0.14 ghij
	G3-1	71.66 ± 1.2 ij	9.36 ± 0.4 de	5.43 ± 0.17 hijk
	G3-3	82 ± 1.53 defgh	9.76 ± 1.23 cde	7.93 ± 0.67 bcde
	G3-11	85.66 ± 1.2 cdef	8.93 ± 0.65 defg	6.16 ± 0.72 ghi
	KR2-5	90.66 ± 3.38 abcd	9.26 ± 1.16 de	7.6 ± 0.75 cdef
	KR3-10	84.66 ± 2.6 cdef	11.5 ± 0.55 ab	7.96 ± 0.4 bcde
	A2-22	91 ± 0.58 abcde	9.36 ± 0.72 de	7.23 ± 0.14 efg
	A2-25	79 ± 2.52 fghi	9.9 ± 0.47 bcde	6.23 ± 0.46 ghi
Non-effective PGPR	G2-22	72.33 ± 2.94 hij	6.9 ± 0.47 h	5.03 ± 0.08 ijkl
	KR1-23	79 ± 2.31 fghi	7 ± 0.11 gh	4.76 ± 0.51 jkl
	KR2-15	66.67 ± 3.24 jk	8.26 ± 1.56 efgh	4.96 ± 1.06 ijkh
	A1-9	65.33 ± 3.23 jk	7.26 ± 0.14 fgh	4.1 ± 0.17 l
	A1-12	71.66 ± 6.36 ij	8.36 ± 0.72 efgh	5.26 ± 0.18 hijkl
	A1-34	80.33 ± 2.06 efghi	8.63 ± 0.49 efgh	5.1 ± 0.32 hijkl

Values with different letters are significantly different according to the LSD test at $P < 0.05$.

agents and biofertilizers have been conducted (Cazorla et al., 2007; Cao et al., 2011; Li et al., 2012; Chen et al., 2013). Identification, screening and eventually selecting the most effective *Bacillus* isolates which control plant disease and enhance crop yield hold great promise in maintaining the sustainability of agroecosystems.

In this work, 303 *Bacillus* isolates were screened by dual culture and volatile metabolite tests. Among them, 20 isolates were identified as highly effective *Bacillus* isolates because they could limit the radial growth of *Fol* considerably. This antagonistic effect may be attributed to the production of diffusable and antifungal compounds such as cyclic peptides (e.g. iturin) and macrolactones including plipastatins, fengycins and surfactins. (Chaurasia et al., 2005; Cao et al., 2011; Gong et al., 2015).

In case of taxonomy characterization of *Bacillus* bacteria, sequence analysis of 16S rRNA accompanied with one of *gyrA*, *gyrB*, *polC* and *rproB* genes can provide conclusive evidence on phylogenetic relationship of *Bacillus* spp. (Chun and Bae, 2000). Therefore, the taxonomy of 20 effective biocontrol isolates was characterized by physiological as well as 16S rRNA gene and *gyrA* gene-based phylogenetic analysis. The results of the physiological tests were in agreement with the results of 16S rRNA gene and *gyrA* gene sequence analysis.

In this study, A1-34 belonged to *B. cereus* species. This isolate was grouped as an effective biocontrol agent because it controlled the *Fusarium* wilt disease by 44.44%. The biocontrol potential of *B. cereus* can be attributed to the production of plipastatins that prevents phospholipase A2 (PLA2) enzyme released by fungal pathogen. This enzyme plays an important role in hydorylizing lipids and phospholipids of host cell (Kohler et al., 2006). Besides, this species enhances defense related phenolic compounds activities (Shafi et al., 2017). Wang et al. (2014) showed that treatment of loquat fruit with *B. cereus* AR156 resulted in lower disease incidence due to the enhancement of defense-related activities such as phenylalanine ammonia-lyase, peroxidase and polyphenoloxidase, chitinase, β-1,3-glucanase, and the stimulated condensing of H_2O_2.

According to the results of present study, G1-8, G2-21, G3-3 and KR2-5 were grouped as effective biocontrol agent because they suppressed *Fusarium* wilt disease between 22% to 27%. Moreover, G3-3 and KR2-5 were classified as effective PGPR agensts because the growth parameters of treated tomato plants with these isolates were close to the growth parameters of non-infected control plants. These isolate were identified as *B. velezensis*. Suppressing the phytopathogenic fungi by *B. velezensis* may be due to the production of diffusable and volatile antifungal compounds. Lim et al. (2017) identified bacillomycin L and fengycin A as agar-diffusible antifungal metabolites produced by *B. velezensis* G341. Moreover, they found that this strain produced three volatile compounds including dimethylsulfoxide, 1-butanol, and 3-hydroxy-2-butanone (acetoin) against various phytopathogenic fungi. Moreover, *Bacillus* strains promoted plant growth by producing phytohormones. *B. velezensis* strain BAC03, enhanced the growth of nine plants including beet, carrot, cucumber, pepper, potato, radish, squash, tomato, and turnip by producing indole-3-acetic acid and ammonia with ACC (1-aminocyclopropane-1-carboxylate) deaminase activity (Meng et al., 2016).

In the present study, KR1-2, KR2-7 and A2-9 isolates characterized as *B. subtilis* remarkably suppressed *Fol* and resulted in the lowest disease index (16.67%) and highest bicontrol efficacy (80%). The biocontrol function of *B. subtilis* encompasses various modes of actions. First, these bacteria induce the systematic resistance in plants (Ongena et al., 2007). Second, they compete for nutrients and ecological niche in rhizosphere environment (Bais et al., 2004). Third, they produce extracellular enzymes such as chinitase and β-1,3-glucanase (Zhang et al., 2008; Chen et al., 2010), a diverse range of antimicrobial compounds, for instance iturin, surfactin and fengysin (Stein, 2005; Baysal et al., 2013), and siderophores which provides iron for plants and deprives fungal pathogen from Iron (Rana et al., 2011). Forth, *B. subtilis* strains form biofilm which serves as biobarrier on the root to protect the plant from pathogen infection (Bais et al., 2004; Morikawa et al., 2006). Since *B. subtilis* possesses these beneficial characteristics, numerous studies were performed to identify new *B. subtilis* isolates and evaluate their biocontrol effectiveness against various ranges of plant pathogens. The application of strain APPL-1 of *B. subtilis* on bean 2 to 120 h before the inoculation of *Uromyces appendiculatus* decreased the number of pustules of bean rust by 95% (Baker et al., 1983). Li et al. (2012) characterized B068150 as *B. subtilis* with biocontrol effect up to 50.68% against *Fusarium oxysporum* f. sp. *cucumerinum* under greenhouse conditions.

Bacillus subtilis is considered as a plant growth promoting rhizobacteria (PGPR) due to its ability in the production of stimulating phytohormones, i.e. indole acetic acid (IAA), gibberellins, and cytokinins, solubilization and mobilization of phosphate, fixation

of nitrogen, siderophores production, inhibition of plant ethylene synthesis and induction of plant systemic resistance to pathogens (Gutierrez-Manero et al., 2001; Whipps, 2001; Idris et al., 2007; Richardson et al., 2009). Inoculation of *Bacillus subtilis* S25 strain on tomato rhizosphere resulted in significant increase in plant growth parameters (Sharma et al., 2015).

This study led to the identification of promising *B. subtilis* isolates, KR1-2, KR2-7 and A2-9, as potentially effective biocontrol agents. Further investigation is needed to elucidate the underlying mechanisms of their antagonistic effect.

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