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Full Length Article

Isolation and Identification of Four Novel Biocontrol *Bacillus* Strains against Wheat Sharp Eyespot and their Growth-Promoting Effect on Wheat Seedling

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Abstract

Natural resistance of wheat plant to sharp eyespot disease is inadequate and new strategies are urgent desired to control this serious soil-borne disease. Biological control is an alternative and attractive approach to effectively reduce the utilization of chemicals in agriculture. In this study, four biocontrol bacterial strains exhibited strong antagonistic activities against *Rhizoctonia cerealis* were isolated from rhizosphere soil of infected wheat *via* a dual culture method. The strains of TA28, TA31, Z-5 and Z-7 were identified as *Bacillus methylotrophicus*, *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis*, respectively, according to morphological, physiological and biochemical characterizations and 16S rRNA gene sequencing. The feasible mechanism of impeding mycelial growth for these *Bacillus* strains was irregularly swollen-tipped and increasing compartment in hypha. The antifungal spectrum indicated that these four strains conferred significant antagonistic effects against many soil-borne pathogenic fungi, including *Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora sojae*. In addition, the bacterium suspension of each strain could actively promote wheat seedling growth. Therefore, the present work provides some new insights on potential candidates for biological control of wheat sharp eyespot and other common soil-borne plant diseases. © 2019 Friends Science Publishers

Keyword: Antagonistic experiment; *Bacillus* spp.; Biological control; Wheat sharp eyespot

Introduction

Wheat sharp eyespot, caused by *Rhizoctonia cerealis* anastomosis group D subgroup I (AG-DI), is a serious wheat stem-base disease in temperate regions worldwide (Li *et al.*, 2017). In infection process, the pathogenic fungus destructs stem and sheath of wheat plant, triggering to block the transportation of nutrients required for the wheat growth (Zhu *et al.*, 2015). Actually, sharp eyespot has been considered as one of the most economically important wheat diseases in China and exceeded approximately an annual loss of million ha in wheat production (Rong *et al.*, 2016).

The main control strategy against *R. cerealis*, in general, is fungicide seed coating and spraying at early jointing stage (Chen *et al.*, 2013). Unfortunately, the undesirable environmental and ecological consequences of increasing fungicide residues have seriously challenged the utilization of chemical agents in agriculture (Talhinhas *et al.*, 2018). Moreover, although cultivars resistant to sharp eyespot can be used to prevent this disease, few wheat cultivars possess moderate resistance and highly resistant cultivars are rare (Wu *et al.*, 2017). Therefore, an environmental-friendly approach has been desired to efficiently control this wildscale soil-borne disease.

Recently, more attention has been drawn to the biological control, which plays an important role in reducing potential environmental and health risks of chemical fungicides and provides an option for combating with soil-borne disease pathogens in crop (Postma *et al.*, 2003; Compant *et al.*, 2005). Thus, it is essential to explore biocontrol agents with high efficiency. Beneficial bacteria are capable of antagonizing pathogens by producing extracellular antifungal compounds and indirectly stimulating the self-defense system of host plants (Beneduzi *et al.*, 2012; Guardado-Valdivia *et al.*, 2018). The *Bacillus* species serve as one of the most realized biocontrol agents on account of a series of connected modes of action and formation of compression resistance with their superior survival ability at different environmental conditions (Setlow, 2014). This favorable feature contributing to antifungal activity is the secretion of active secondary metabolites, including low molecular weight volatile compounds and antibiotics (Manns *et al.*, 2012; Wang *et al.*, 2014a; Wu *et al.*, 2015). Additionally, as an important genus, *Bacillus* strains are able to produce multiple

antimicrobial peptides with considerable characteristics such as strong antifungal activities, good biodegradability, wider antimicrobial spectrum and high temperature tolerance (Meena and Kanwar, 2015).

In the current study, four different *Bacillus* isolates were identified and their antagonistic activity against several soil-borne pathogenic fungi, especially to *R. cerealis*, was studied.

Materials and Methods

Isolation of Bacterial Strains

Shandong province is one of the major wheat producing districts in China that has been subjected to sharp eyespot decease for decades (Zhang *et al.*, 2017). Six main wheat-growing areas in this province including Liaocheng, Heze, Dezhou, Tai'an, Linyi and Laizhou were selected for soil collection. Samples were collected from the upper 10–15 cm of the soil profile, and each sampling site was georeferenced. Approximately 10 g of soil sample from each sit was obtained and subsequently suspended with 90 mL of sterile distilled water. After 5-fold serial dilution, 200 μL of the resultant suspension was spread out on nutrient agar (NA) plates and incubated at 28°C for 48 h. Colonies generated on NA plates were inoculated onto Luria-Bertani (LB) agar plates for identification and preservation.

Antagonistic Activity Assay

Three hundred bacterial strains were isolated from collected soil samples. After then, antagonism of the isolates was performed *in vitro* against *R. cerealis* through a direct dual culture method as previously reported by Oldenburg *et al.* (1996). Each fungus cake of 0.5 cm in diameter was placed in the centre of potato dextrose agar (PDA) plate and 20 μL (1×10^8 CFU/mL) of the bacterial suspension was inoculated to the periphery of agar surface. After incubation at 28°C for three days, the antagonistic activity was detected by measuring the inhibition zones and the colony diameters. The percentage of growth inhibition was calculated using the formula of $y= (a-b)/a\times100$, where y means the percentage growth inhibition; a means the colony area of uninhibited *R. cerealis*; b means the colony area of treated *R. cerealis* (Mikani *et al.*, 2008). The marginal hypha around the inhibition zone was used to detect the morphological change, which was subjected to the antifungal substance secreted from the bacterial isolate.

Three common kinds of soil-borne disease pathogenic fungi were also used to assess the antagonistic effect of these bacterial isolates, including *Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora sojae*, following the above described method. *R. cerealis* strain WK-207 used in this study was isolated from wheat samples symptomatic for sharp eyespot at Shandong province in China (Ji *et al.*, 2017). All of these mentioned pathogenic fungi were donated by prof. Aixin Liu from Shandong Agricultural University. The cell concentration was estimated using a hemocytometer. All experiments were conducted in triplicate.

Identification of Strains

Identification was performed by conventional methods based on physiological and biochemical reactions according to the Bergey's manual of systematic bacteriology (Pandya and Saraf, 2015) and further confirmed by molecular characterization. Total DNA was prepared by phenolic extraction and isopropyl alcohol precipitation (Llop *et al.*, 1999). The gene sequence of 16S rRNA was amplified using a pair of specific primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Yu *et al.*, 2013). PCR reaction program was as follows: 94°C, 5 min; (94°C, 30 s; 55°C, 30 s; 72°C, 60 s) of 30 cycles; 72°C, 10 min. The amplified product was connected to pMD18-T vector (TaKaRa Biotechnology Co. Ltd., China) and the recombinant plasmid was transformed to *E. coli* T1 (TransGen Biotech, Beijing, China). The positive transformant was gene sequenced to confirm the cloned gene was correct. Gene synthesis and sequencing was conducted by Sangon Biotech (Shanghai, China). DNA sequences were analyzed using the BLAST program and constructed a phylogenetic tree with relative bacteria strains using the neighbor-joining method built in the MEGA 7 software (Wang *et al.*, 2014b).

Growth-promoting Test on Wheat Seedlings

A pot experiment was performed to detect the growth-promoting effect on wheat seedlings in a greenhouse with temperatures ranged from 25°C to 30°C in daytime and 15 to 20°C during night. The relative humidity was modulated between 60 and 80%. Seeds of susceptible wheat cultivar Taishan 9818 were used and six seeds per pot were sown in triplicate. Two hundred milliliter of culture supernatant with 1×10^8 CFU/mL was used to root-irrigation at the time of seedling emergence and continuous irrigating for 3 times every ten days. Then, seed growth parameters were measured, including plant height, root length and the number of roots (Li *et al.*, 2013).

Results

Screening of Isolates with Antagonistic Test

Four strains with the considerable inhibited effect were selected and named as TA28, TA31, Z-5 and Z-7 (Fig. 1). The average percentage of growth inhibition to *R. cerealis* for each strain exceeded 50% (Table 1). Besides, four isolated strains exhibited antagonistic activity to three kinds of common soil-borne disease pathogenic fungi at different

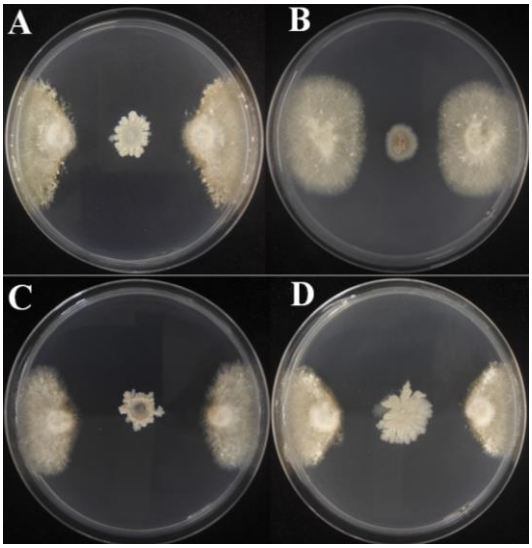

Fig. 1: Antagonistic activity of four bacterial isolates in dual culture study against fungal pathogens *Rhizoctonia cerealis* on PDA plates. **A**: the TA28 strain; **B**: the TA31 strain; **C**: the Z-5 strain; **D**: the Z-7 strain

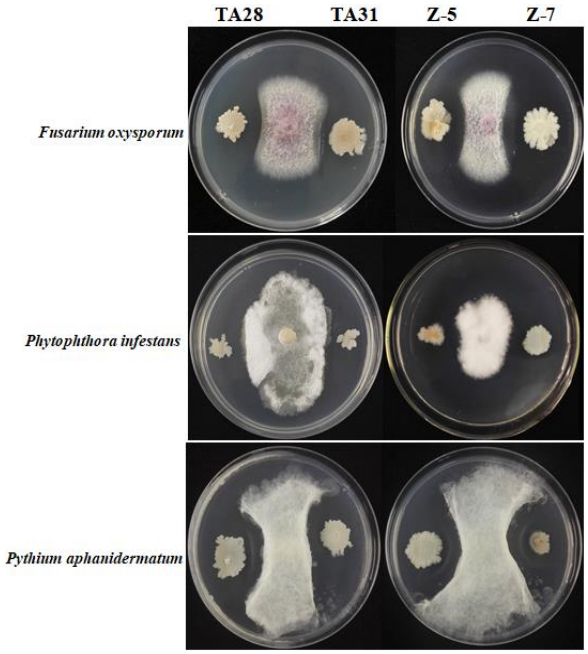

Fig. 2: Four bacterial isolates showed significant antagonistic activities against three common soil-borne phytopathogenic fungi on PDA plates

degrees (Fig. 2), including *F. oxysporum*, *P. aphanidermatum* and *P. sojae*, which usually caused Fusarium wilt, Pythium root rot and Phytophthora blight in various plants, respectively. The isolate Z-7 had a significant antifungal effect on *F. oxysporum* as the inhibition was 85.9%, but lower activity on *P. aphanidermatum* of 46.9%. However, the other isolates showed an obvious antifungal effect on these pathogenic fungi following the order of *P. sojae* > *R. cerealis* > *F. oxysporum* > *P. aphanidermatum* (Table 1). These results suggested that these isolates could evidently inhibit the growth of soil-borne disease pathogenic fungi.

Table 1: Inhibition of mycelial growth of soil-borne phytopathogenic fungi by four bacterial strains

Phytopathogenic fungi	Inhibition of mycelial growth (%)			
	TA28	TA31	Z-5	Z-7
Rhizoctonia cerealis	61.0 ± 4.6	53.3 ± 3.0	75.0 ± 2.1	66.3 ± 2.9
Fusarium oxysporum	55.6 ± 1.2	67.7 ± 1.8	66.7 ± 0.6	85.9 ± 1.6
Phytophthora sojae	81.1 ± 0.8	75.7 ± 1.1	82.2 ± 0.5	72.2 ± 0.7
Pythium aphanidermatum	50.0 ± 1.1	53.1 ± 0.9	54.3 ± 1.0	46.9 ± 1.5

Percentage growth inhibition was calculated using the formula $y = (a-b)/a \times 100$. y is the percentage growth inhibition; a is the colony area of the uninhibited phytopathogenic fungus; b is the colony area of the treated fungus. Values are means ±SD of three replications

Hyphal Morphologic Change of *R. cerealis*

The hyphal morphologic change of *R. cerealis* was detected using the marginal hypha around the inhibition zone after confront culture for three days. These four isolates exhibited similar antagonistic effect on morphologic change of *R. cerealis* hypha. As shown in Fig. 3, the hyphal growth was inhibited as the malformation at top cells compared with the normal hyphal growth. Hyphal cells were excessive sunken and intumescentia, which caused the incremental quantity of branches. Consequently, the malformed cells were separated from other parts of hypha. This result revealed that the probable mechanism of antagonistic action on *R. cerealis* was the hyphal malformation ascribed to antifungal substances which were secreted from bacterial isolates.

Identification of Isolations

Based on the Bergey's manual of systematic bacteriology, physiological and biochemical characterizations were performed and the result was illustrated at Table 2. All these properties indicated that these four strains belong to *Bacillus* species. However, some different properties among these bacterial strains, for example, viscosity, available carbon sources, tolerance to NaCl, pH range for growth etc., suggesting that they were not the same kind of *Bacillus* species. This conclusion was clarified by 16S rRNA gene sequencing, which has been previously demonstrated to effectively recognize prokaryotes with close evolutionary relationships. GenBank accession numbers of 16S rRNA sequences of TA28, TA31, Z-5 and Z-7 were KT253248, KU361185, KF836532 and KX129847, respectively. After amplifying and aligning of 16S rRNA genes for each strain, a phylogenetic tree was constructed through the neighbor-joining method (Fig. 4). TA28 exhibited a high degree of similarity to *B. methylotrophicus* strain HB26 (KM659227) with 99% sequence identify, indicating that the TA28 strain belonged to the species *B. methylotrophicus.*

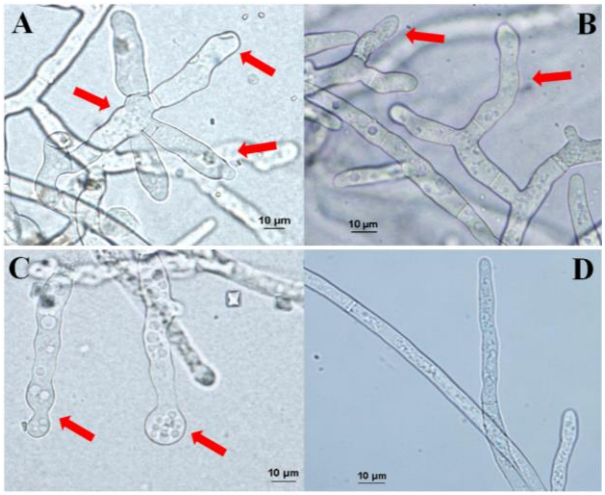

Fig. 3: Hyphal morphological change of *Rhizoctonia cerealis* antagonized with bacterial isolates. **A**, **B** and **C**: *R. cerealis* treated with bacterial suspension culture; **D**: the normal hyphal growth as control. The red arrow means the swollen-tipped and increasing compartment in deformed hypha

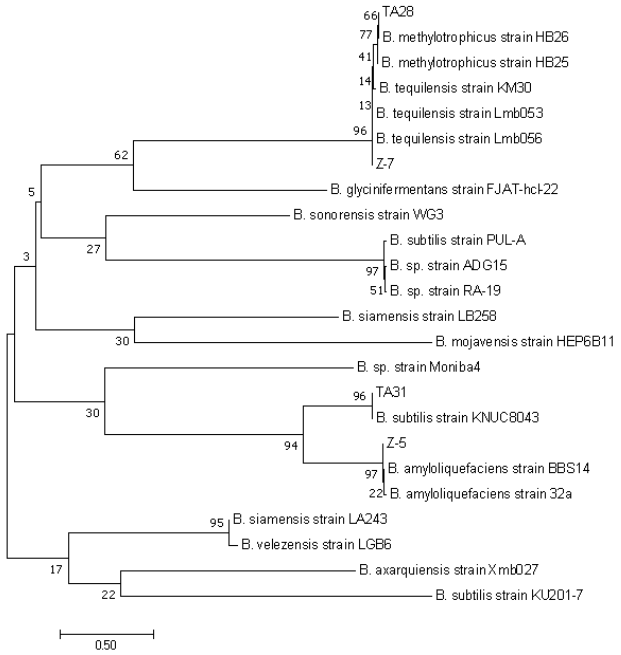

Fig. 4: Rooted neighbor-joining tree based on 16S rRNA gene sequencing. The percentage numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. The scale bar indicates 0.50 nucleotide substitutions per nucleotide position

Similarly, Z-7, TA31 and Z-5, sharing 100, 99 and 100% sequence identify with *B. tequilensis* strain Lmb056 (KT986121), *B. subtilis* strain KNUC8043 (KU534102) and *B. amyloliquefaciens* strain BBS14 (KC887504), were identified as *B. tequilensis*, *B. subtilis* and *B. amyloliquefaciens*, respectively.

Table 2: Physiological and Biochemical characteristics of these bacterial strains

Characteristics	TA28	TA31	Z-5	Z-7
Color of colony	white	light yellow	light yellow	white
Margin	irregular	regular	irregular	irregular
Transparence	-	-	-	-
Cell shape	rod-shaped	rod-shaped	rod-shaped	rod-shaped
Gram stain	G^+	G^+	G^+	G^+
Fluorescence	-	-	-	-
Motility	+	-	+	-
Viscosity	-	-	+	+
Methyl red test	-	+	+	-
Voges-Proskauer test	+	+	+	+
Anaerobic growth	+	-	+	-
Hydrolysis of mannose	-	+	+	-
Hydrolysis of xylose	+	-	-	+
Utilization of citrate	+	+	+	+
Catalase activity	+	+	+	+
Tolerance to NaCl	<15%	<10%	<10%	<15%
Range of growth pH	5-8	7-8	6-9	7-9

Morphological characteristics were detected using NA plates as culture medium

"+" means positive; "-" means negative

Pot Experiment

The growth-promoting effect of bacterial isolates on wheat seedlings was detected using the pot experiment. Compared to the control, a drastic promotion of growth vigour was observed with each treatment (Fig. 5). Stem heights were increased to 1.33-, 1.43-, 1.34- and 1.36-fold for TA28, TA31, Z-5 and Z-7 treatment, respectively. Besides, root lengths were also enhanced to different degrees by these *Bacillus* strains as shown in Table 3. The maximum stimulatory effect on seedling roots was recorded with the treatment of TA28. Results revealed that wheat seedlings treated with TA28 showed significantly more main roots and fibrous roots than the non-inoculated control.

Discussion

As an effective approach to control plant diseases, biological control creates a long-term effect to facilitate sustainable agriculture (Sylla *et al.*, 2013). Although multiple bacterial strains with strong antagonistic activities against soil-borne plant diseases have been isolated and commercialized, more effective candidates are desired to prevent the sharp eyespot disease in wheat. In order to obtain potential biological control agents, the dual culture method *in vitro* is considered as a crucial step of screening strategy (Li *et al.*, 2013).

In this study, four biocontrol bacterial strains, signed as TA28, TA31, Z-5 and Z-7, were isolated from rhizosphere soil of infected wheat. These strains possessed considerable antagonistic activities against pathogenic *R. cerealis* and effectively inhibited the mycelial growth of other kinds of plant pathogenic fungi, including *F. oxysporum*, *P. aphanidermatum* and *P. sojae*. Actually, the broad-spectrum antifungal property is an inherent feature of some biocontrol bacteria, which could be

Table 3: Growth-promoting effects of bacterial strains on wheat seedlings

Treatment	Stem height (cm)	Root length (cm)	Average number of main roots	Relative quantity of fibrous roots
Control	16.3 ± 0.8^{a}	11.3 ± 1.0^{a}	3.2^{a}	+
TA28	21.7 ± 0.3^{b}	14.7 ± 0.6^{b}	5.6^{c}	+++
TA31	22.3 ± 1.0^{b}	15.1 ± 0.8^{b}	4.6^{b}	++
Z-5	21.8 ± 0.8^{b}	14.5 ± 1.5^{b}	5.2^{bc}	++
Z-7	22.2 ± 1.5^{b}	17.4 ± 1.0^{c}	4.6^{b}	++

200 mL of strain culture supernatant with 1×10^{8} CFU/mL was used for root-irrigation at the time of seedling emergence and continuous irrigating for 3 times every ten days. 200 mL of sterile LB culture solution was used as control and irrigated wheat seedlings in the same condition as treatment. Statistical analysis was conducted using the Student-Newman-Keuls (SNK) method. Different letters in same column represent statistically significant differences ($P < 0.05$). "+" means 0-20; "++" means 20-40; "+++" means 40-60. Values are means ±SD of three replications

potential commercial candidates of raw material for an effective biocontrol agent (Dharni *et al.*, 2012). As previously reported, *Bacillus* strain RMB7 inhibited >70% growth of nine fungal phytopathogens *in vitro* and the bacterial supernatant exhibited a resistance against phytopathogenic *Pythi*um *irregulare* with a concurrent plant growth improvement compared with non-inoculated plants (Ali *et al.*, 2014). In addition, *Burkholderia cepacia* MPC-7 markedly reduced the wilting rate of pepper plant caused by *Phytophthora capsic*. More importantly, MPC-7 demonstrated a broad-spectrum antimicrobial activity against several pathogenic fungi and bacteria (Sophearet*h et al.*, 2013).

These isolates displayed a beneficial effect on the suppression of pathogenic fungi. The mechanism of biological control is probably attributed to the production of antifungal substances, which cause excessive hyphal malformation at top cells and accelerate the hyphal separation. Biocontrol bacteria, especially *Bacillus* species, often secrete bacilysin and iturin as important compositions in antagonistic effect (Compaoré *et al.*, 2013). Alternatively, cell wall-degrading enzymes (CWDES) and lipopeptid could further interfere with the fungal growth in antifungal compound processes (Gond *et al.*, 2015; Khaledi *et al.*, 2015). However, the exact mechanism involved in the antagonistic activity of these four isolates should be elucidated by in-depth research.

To identify species of these bacteria, morphological, physiological and biochemical characteristics and 16S rRNA gene sequencing were conducted. Consequently, the strains of TA28, TA31, Z-5 and Z-7 were identified as *B. methylotrophicus*, *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis*, respectively. All these isolated *Bacillus* strains possessed desirable antifungal activities to various soil-borne phytopathogenic fungi. Similar conclusion was reported on other *Bacillus* strains, which were initially screened using a dual culture and obviously inhibited the hyphal growth of pathogenic fungi (Solanki *et al.*, 2015). *B. amyloliquefaciens* GR53 resists *Rhizoctonia* disease on Chinese cabbage through hormonal and antioxidants regulation, which effectively mitigated *R. solani*-induced damages and improved plant growth (Kang *et al.*, 2015). Besides, *B. subtilis* NJ-18 has a broad-spectrum antimicrobial activity to phytopathogenic fungi. When the NJ-18 strain was cooperated with jinggangmycin (the most widely used fungicide to control rice sheath blight in China), the synergetic suppression of this disease was improved (Peng *et al.*, 2014). As a result, these four isolates provide alternative soil-borne plant disease control options.

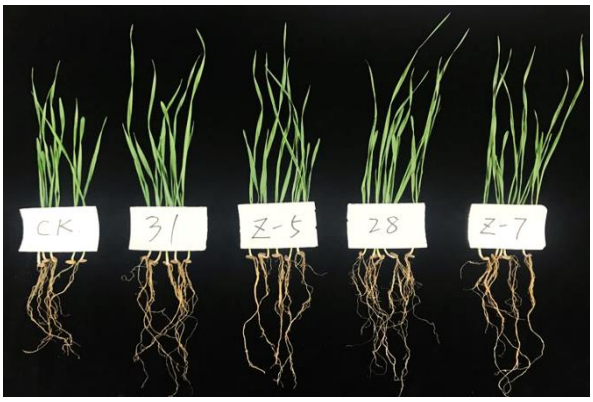

Fig. 5: The growth-promoting effect of each isolate on wheat seedlings. 200 mL of sterile LB culture solution was used as control

In the present experiment, the isolate TA28 was able to promote the wheat seedling growth with an impressive increase of stem height, root length and the quantity of roots. Other isolates also enhanced the seedling growth at different degrees. Evidently, some *Bacillus* strains are regarded as important compositions of plant growth-promoting rhizobacteria (PGPR), which is a group of microbes colonizing the roots and stimulating plant growth either directly or indirectly (Cao *et al.*, 2018). The dominant plant growth modulators produced by *Bacillus* strains were volatile organic compounds (VOCs), such as 2,3-butanediol and 3-hydroxy-2-butanone (Rath *et al.*, 2018). Additionally, VOCs can mediate induce systemic resistance (ISR) in response to pathogen challenges in several plant species (Velázquez-Becerra *et al.*, 2011). These four strains may share the same growth-promotion mechanism employed by rhizospheric bacteria which generated VOCs production.

Conclusion

Four new biocontrol bacterial strains, which possessed effective antagonistic activity against *R. cerealis*, were isolated *via* a direct dual culture method. Based on

morphological, physiological and biochemical characteristics and 16S rRNA gene sequencing, isolated strains of TA28, TA31, Z-5 and Z-7 were identified as *B. methylotrophicus*, *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis*, respectively. These *Bacillus* strains exhibited remarkable antifungal action against several soil-borne pathogens and actively promoted the wheat seedling growth. These properties are essential for biocontrol agents and make these strains act as interesting potential candidates for biological control of wheat sharp eyespot and other common soil-borne plant diseases.

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