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GENOME REPORTS

Draft genome sequence of *Bacillus paralicheniformis* TRQ65, a biological control agent and plant growth-promoting bacterium isolated from wheat (*Triticum turgidum* subsp. *durum*) rhizosphere in the Yaqui Valley, Mexico

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Abstract

The strain denominated TRQ65 was isolated from wheat (*Triticum turgidum* subsp. *durum*) commercial fields in the Yaqui Valley, Mexico. Here, we report its draft genome sequence, which presented ~ 4.5 million bp and 45.5% G + C content. Based on the cutoff values on species delimitation established for average nucleotide identity (> 95 to 96%), genome-to-genome distance calculator (> 70%), and the reference sequence alignment-based phylogeny builder method, TRQ65 was strongly affiliated to *Bacillus paralicheniformis*. The rapid annotation using subsystem technology server revealed that TRQ65 contains genes related to osmotic, and oxidative stress response, as well as auxin biosynthesis (plant growth promotion traits). In addition, antiSMASH and BAGEL revealed the presence of genes involved in lipopeptides and antibiotic biosynthesis. The function of those annotated genes was validated at a metabolic level, observing that strain TRQ65 was able to tolerate saline (91.0%), and water (155.0%) stress conditions, besides producing 28.8 ± 0.9 µg/mL indoles. In addition, strain TRQ65 showed growth inhibition (1.6 ± 0.4 cm inhibition zone) against the causal agent of wheat spot blotch, *Bipolaris sorokiniana.* Finally, plant–microbe interactions assays confirm the ability of strain TRQ65 to regulate wheat growth, showing a significant increment in shoot height (26%), root length (40%), shoot dry weight (48%), stem diameter (55%), and biovolume index (246%). These findings provide insights for future agricultural studies of this strain.

Keywords Plant growth-promoting rhizobacteria · Biocontrol agent · Average nucleotide identity · Genome to genome distance calculator · Biofertilizer

Introduction

The genus *Bacillus* was first isolated and described as a rod-shaped, heat-resistant, and endospore-forming Gram-positive bacterium (Cohn 1872). The species of this genus are widely distributed due to their ability to form endospores, which provide them resistance to several habitats, such as: environments under optimal or extreme conditions (Tejera-Hernández et al. 2011). Soil is considered the main reservoir of *Bacillus,* due to the great metabolic diversity of this genus associated with metabolizing a large source of organic compounds (McSpadden Gardener 2004).

In agriculture, the *Bacillus* species are the most extensively studied bacteria for (1) controlling/inducing plant systemic resistance against phytopathogens, by consumption of leached exudates, production of siderophores,

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activity of lytic enzymes (chitinases, glucanases, proteases), production of antibiotics, and biosynthesis of cyclic lipopeptides (Villarreal-Delgado et al. 2017; Tiwari et al. 2019), and (2) promoting plant growth and development, through the production or regulation of phytohormones, solubilization of phosphates, activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, production of siderophores, and biological nitrogen fixation (Berendsen et al. 2012; Trabelsi and Mhamdi 2013; Barra et al. 2015; García-Meléndez et al. 2017; Valenzuela-Ruiz et al. 2018; Robles-Montoya et al. 2019). Thus, *Bacillus* has been used as an active ingredient for the formulation of (1) biofungicides, *v.gr.* Ballad Plus (*Bacillus pumilus* QST2808 against *Erysiphe,* and *Puccinia*), Serenade ASO (*B. subtilis* QST713 against *Pythium, Rhizoctonia,* and *Fusarium*), Fungifree AB (*B. subtilis* 83 against *Colletotrichum,* and *Leveillula*), EcoGuard-GN (*Bacillus licheniformis* SB3086 against *Colletotrichum,* and *Sclerotinia*), and DiPel WG (*Bacillus thuringiensis* against *Cydia,* and *Otiorhychus*) (Villarreal-Delgado et al. 2017; Villa-Rodríguez et al. 2019), and (2) biofertilizers, *v.gr*. BIOXTERRA BS (*Bacillus subtilis*), BIOXTERRA BT (*Bacillus thuringiensis*), Bio-P (*Bacillus subtilis*, and *Azotobacter chroococcum*), and Hydroguard (*Bacillus amyloliquefaciens*), which colonize and protect the rhizosphere improving root biomass and vigor of plants (Botanicare 2012; Valenzuela-Aragon et al. 2018; Kashyap et al. 2019; BioAgro Chemical 2019a, b; AGSOL 2019).

Strain TRQ65 was isolated from wheat (*Triticum turgidum* subsp. *durum*) rhizosphere of commercial fields, in the Yaqui Valley, Mexico (27.3692°, 110.3886°). This strain is preserved in Colección de Microorganismos Edáficos y Endófitos Nativos (México) (COLMENA, http://www.itson.edu.mx/COLMENA) (de los Santos Villalobos et al. 2018). According to the strong association of TRQ65 with wheat plants in the field, we inferred a synergistic interaction between them, which needs to be studied to propose its potential use in the biocontrol of phytopathogens that affect wheat production and/or to regulate the growth of this crop. Previously, Valenzuela-Aragon et al. (2018) and Villa-Rodríguez et al. (2019)—based on the 16S rRNA sequencing—affiliated the strain TRQ65 to the genus *Bacillus*; however, Diaz-Rodriguez et al. (2019) affiliated this strain to *Bacillus licheniformis*. Since it is one of the largest bacterial genera [comprising 377 named species and 7 subspecies, including synonyms (Parte 2018), with a great genomic and metabolic diversity], its taxonomic affiliation is complex on the basis of traditional phenotypic (Fan et al. 2017) and molecular methods (sequencing of the 16S rRNA gene) (Rooney et al. 2009). Thus, the genome of TRQ65 was sequenced to (1) clarify its taxonomic affiliation and (2) explore its genomic and metabolic background associated with biological control of phytopathogens and wheat growth promotion.

High-quality genomic DNA was extracted from a fresh culture of strain TRQ65, which was grown in Nutrient Broth [24 h at 32 °C, using an orbital shaker at 121 rpm, obtaining 1×10^6 colony forming units (CFU)/mL], and following the protocol described by Valenzuela-Aragon et al. (2018). Then, the bacterial DNA was sequenced by Illumina MiSeq platform, obtaining a total of 5,079,308 total reads [2×300 base pairs (bp)]. The quality of the obtained reads was analyzed by FastQC version 0.11.5 (Andrews 2010). Trimmomatic version 0.32 (Bolger et al. 2014) was used to remove adapter sequences and low-quality bases, and only 8.42% was dropped. Subsequently, de novo assembly was generated by SPAdes version 3.10.1 (Bankevich et al. 2012), using the "–careful" parameter for error correction in reads. The draft genome of TRQ65 presented 4,475,481 bp; 45.5% G + C content; 676,421 bp N50; 3 L50; and 32 contigs (> 200 bp). The assembled contigs were ordered by Mauve contig Mover version 2.4.0 (Darling et al. 2004), using the reference genome of *Bacillus paralicheniformis* KJ-16^T [KY694465]. In addition, the presence of plasmids in the TRQ65 genome was analyzed by PLACNETw (https://castillo.dicom.unican.es/upload/) (Vielva et al. 2017); however, no plasmids were observed for strain TRQ65, and to our understanding the presence of plasmids has not been reported for this species.

The 16S rRNA gene sequence of TRQ65 was used to confirm the authenticity of the studied genome according to Chun et al. (2018). In addition, the gene sequence was submitted to NCBI and EzBioCloud database to determine the more closely related strains (based on the cutoff values on species delimitation established for the 16S rRNA gene > 98.7%) (Yoon et al. 2017a; Chun et al. 2018). Thus, the highest similarity values (100%) for the 16S rRNA gene sequence of TRQ65 corresponded to *Bacillus paralicheniformis* KJ-16^T [KY694465], *Bacillus haynesii* NRRL B-41327^T [MRBL01000076], and *Bacillus licheniformis* ATCC 14580^T [AE017333], followed by *Bacillus glycinifermentans* GO-13 ^{T} [LECW01000063], 99.92%, and *Bacillus sonorensis* NBRC 101234^T [AYTN01000016], 99.84% (Table 1). This finding supports the previous taxonomic affiliation of strain TRQ65 to the genus *Bacillus* (Valenzuela-Aragon et al. 2018; Villa-Rodríguez et al. 2019; Diaz-Rodriguez et al. 2019). To affiliate that strain at a species level, its genome was compared to its more closely related strains (Table 1), by using (1) the average nucleotide identity (ANI), by the OrthoANI algorithm (Yoon et al. 2017b) and (2) the Genome to Genome Distance Calculator (GGDC) version 2.1, by BLAST (Meier-Kolthoff et al. 2013). Those bioinformatics tools have been proposed as a strong approach to clarify the taxonomic affiliation of prokaryotes, which has been used to discover a novel *Bacillus* species, *B. cabrialesii* TE3^T (de los Santos-Villalobos et al. 2019). Thus, based on the profound taxonomic affiliation provided by those tools,

Table 1 16S rRNA similarity, ortho average nucleotide identity (ANI), and Genome to Genome Distance Calculator (GGDC) values by the genome comparison of TRQ65 vs. its more closely related species (16S rRNA > 98.7%)

Strain TRQ65 compared to:	16S rRNA similarity	Ortho ANI	GGDC
	%	%	%
Bacillus paralicheniformis KJ-16^T [KY694465]	100.00	99.07	92.40
Bacillus haynesii NRRL B-41327^T [MRBL01000076]	100.00	95.13	61.40
Bacillus licheniformis ATCC 14580^T [AE017333]	100.00	94.56	57.60
Bacillus swezeyi NRRL B-41294^T [MRBK01000096]	99.67	83.27	26.10
Bacillus sonorensis NBRC 101234^T [AYTN01000016]	99.84	81.55	24.70
Bacillus glycinifermentans GO-13^T [LECW01000063]	99.92	80.84	23.70
Bacillus subtilis subsp. *inaquosorum* KCTC 13429^T [AMXN01000021]	98.93	73.11	19.00
Bacillus subtilis subsp. *spizizenii* NRRL B-23049^T [CP002905]	99.01	72.93	19.10
Bacillus nakamurai NRRL B-41091^T (LSAZ01000028)	98.76	72.84	18.70
Bacillus tequilensis KCTC 13622^T [AYTO01000043]	98.93	72.79	18.80
Bacillus atrophaeus JCM9070^T [AB021181]	98.76	72.74	18.60
Bacillus velezensis CR-502^T [AY603658]	98.70	72.73	19.50
Bacillus subtilis subsp. *subtilis* NCIB 3610^T [ABQL01000001]	98.85	72.70	19.00
Bacillus halotolerans ATCC 25096^T [LPVF01000003]	98.85	72.45	18.50
Bacillus siamensis KCTC 13613^T [AJVF01000043]	98.76	72.42	19.00
Bacillus subtilis subsp. *stercoris* D7XPN1^T [JHCA01000027]	98.85	72.39	18.80

at a species level [ANI > 95-96% (Varghese et al. 2015), and GGDC > 70% (Yoon et al. 2017b)], TRQ65 was strongly affiliated to *Bacillus paralicheniformis* (Table 1).

In addition, a phylogenetic tree was constructed to further support the authenticity of the genome data, as well as to determine the genetic relationship between strain TRQ65 and its more closely related species. Thus, the genome sequences were aligned using the reference sequence alignment-based phylogeny (REALPHY) builder method version 1.12 (Bertels et al. 2014), followed by the generation of the genome-based phylogenetic tree by MEGA version 7.0 (Kumar et al. 2016). The neighbor-joining method was used with a bootstrap support of 1000 replications, which confirmed that the taxonomic affiliation of TRQ65 is *Bacillus paralicheniformis* (Fig. 1).

The genome annotation of the studied strain was created through Rapid Annotation Using Subsystem Technology (RAST) server version 2.0 (http://rast.nmpdr.org) (Aziz et al. 2008; Overbeek et al. 2013), by the RASTtk pipeline. Strain TRQ65 showed a total of 91 RNAs, and 4811 predicted coding DNA sequences (CDS)—distributed into 361 subsystems. The most abundant subsystem was amino acids and derivatives (392 genes), followed by carbohydrates (360 genes), protein metabolism (207 genes), cofactors, vitamins, prosthetic groups, and pigments (165 genes), nucleosides and nucleotides (103), and dormancy and sporulation (96) (Fig. 2).

The genome of strain TRQ65 revealed the presence of genes involved in (1) the tolerance to abiotic factors in agrosystems (oxidative and water stress conditions), (2) the biological control of phytopathogens (lipopeptides and antibiotic biosynthesis), and (3) the promotion of plant growth (auxin biosynthesis) (Table S1). Putative annotated genes of strain TRQ65 were validated through a metabolic characterization according to Valenzuela-Aragon et al. (2018). The percentage of the abiotic stress tolerance by TRQ65 was calculated by subtracting the bacterial growth (cm) under abiotic stress condition minus the bacterial growth (cm) under optimal condition, and dividing by the bacterial growth (cm) under optimal condition. TRQ65 showed the ability to grow—compared to control conditions—on Petri dishes containing nutrient agar under saline (sodium chloride 5%, 6.8 dS m^{-1}, for 3 days at 28 °C) stress, 91.0 ± 5.3%, and water (polyethylene glycol 6000 10%, − 0.84 mPa, for 3 days at 28 °C) stress, 155.0 ± 3.7%. Similar findings have been reported by Palacio-Rodríguez et al. (2017), Obeidat (2017), and Rajabi Agereh et al. (2019), associating the tolerance of abiotic stress conditions from bacterial strains to genes involved in glycerol, ferric, iron, and zinc uptake, as well as fumarate and nitrate regulation. Those and other promising genes were found in the TRQ65 genome (Table S1).

On the other hand, antiSMASH version 5.0 (https://antismash.secondarymetabolites.org) and BAGEL version 4.0 (http://bagel4.molgenrug.nl/) were used to identify putative genes in the TRQ65 genome involved in the biological control of phytopathogens. Thus, eight genes associated with lipopeptide biosynthesis, bacitracin, bacillibactin, butirosin, lichenysin, haloduracin alpha, haloduracin beta, were identified by antiSMASH; and nine genes associated with lipopeptide biosynthesis, lichenicidin, haloduracin alpha, bottromycin, enterocin, and sonorensin,

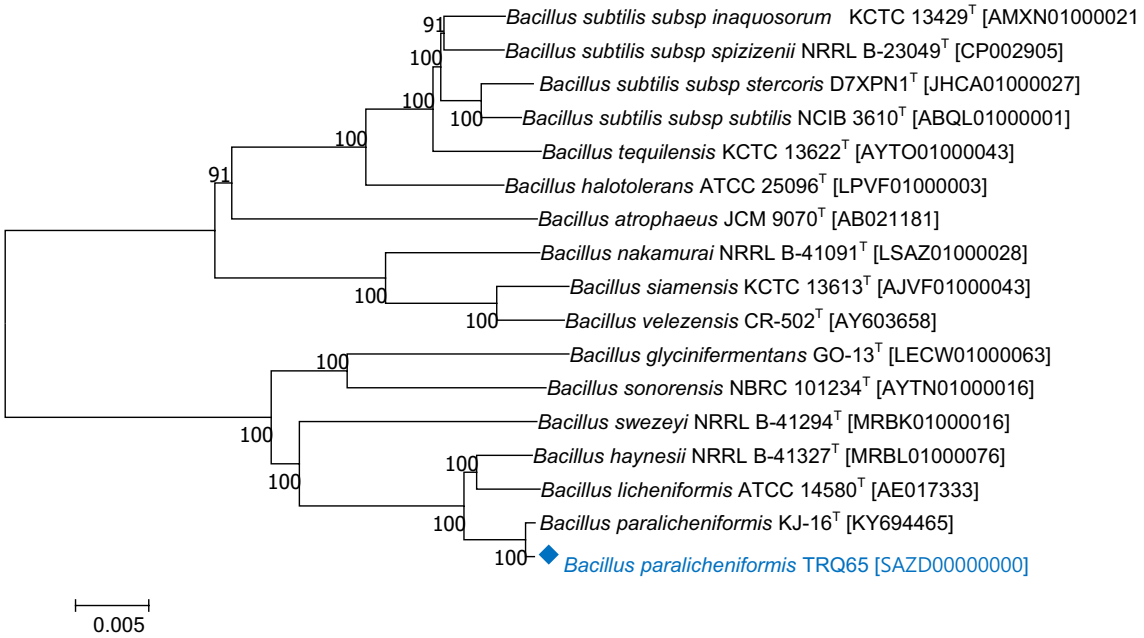

Fig. 1 Phylogenetic relation between TRQ65 and closely related species: *Bacillus paralicheniformis* KJ-16^T [KY694465]; *Bacillus haynesii* NRRL B-41327^T [MRBL01000076]; *Bacillus licheniformis* ATCC 14580^T [AE017333]; *Bacillus glycinifermentans* GO-13^T [LECW01000063]; *Bacillus sonorensis* NBRC 101234^T [AYTN01000016]; *Bacillus swezeyi* NRRL B-41294^T [MRBK01000096]; *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049^T [CP002905]; *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429^T [AMXN01000021]; *Bacillus tequilensis* KCTC 13622^T [AYTO01000043]; *Bacillus subtilis* subsp. *subtilis* NCIB 3610^T [ABQL01000001]; *Bacillus halotolerans* ATCC 25096^T [LPVF01000003]; *Bacillus subtilis* subsp. *stercoris* D7XPN1^T [JHCA01000027]; *Bacillus atrophaeus* JCM 9070^T [AB021181]; *Bacillus nakamurai* NRRL B-41091^T [LSAZ01000028]; *Bacillus siamensis* KCTC 13613^T [AJVF01000043]; *Bacillus velezensis* CR-502^T [AY603658], constructed by the builder method in REALPHY 1.12 (Bertels et al. 2014) and MEGA 7 (Kumar et al. 2016) using the neighbor-joining algorithm (based on 1000 bootstrap replications). Scale bar (0.005) represents the number of nucleotide substitutions per site

were identified by BAGEL (Table S1). These lipopeptides have been reported as having antitumor, immunosuppressant, surfactant, cytotoxic, and antimicrobial properties (Raaijmakers et al. 2010). To validate the functionality of those putative genes in the TRQ65 genome, the antagonistic ability of this strain was evaluated in vitro against *Bipolaris sorokiniana*, the causal agent of wheat spot blotch (Villa-Rodriguez et al. 2016). For this, a three replicate quantitative assay was performed according to Villa-Rodríguez et al. (2019), a volume of 10 µL of *Bipolaris sorokiniana* TPQ3 conidia suspension (1×10^5 conidia/mL) was placed in the center of Petri dishes containing potato dextrose agar, and 10 µL of *Bacillus paralicheniformis* TRQ65 cell suspension (1×10^6 CFU/mL) was inoculated in two equidistant points, at about 2 cm distance of the studied phytopathogen. After an incubation for 5 days at 28 °C, the inhibition halo of *Bipolaris sorokiniana* TPQ3 by strain TRQ65 was quantified. *Bacillus paralicheniformis* TRQ65 showed an inhibition zone of 1.6 ± 0.4 cm against *Bipolaris sorokiniana* TPQ3, which confirms the function of putative genes associated with the biological control of phytopathogens found in the genome of TRQ65 by antiSMASH and BAGEL (Table S1).

Regarding the ability of strain TRQ65 to promote the growth and development of plants, this strain was able to biosynthesize 28.8 ± 0.9 µg/mL indoles through the Salkowski method (Rahman et al. 2010). This finding confirms the functionality of the identified putative genes in the TRQ65 genome associated with the biosynthesis of that phytohormone (Figure S1). In addition, to validate the ability of strain TRQ65 to regulate the growth of plants, an axenic in vivo plant–bacterium interaction assay was performed in a growth chamber, under controlled conditions. Thus, wheat variety CIRNO C2008 seeds were washed three times in sterile distilled water, followed by soaking in 70% (v/v) ethanol for 1 min, washed with 3% (v/v) sodium hypochlorite for 10 min, and five additional washes with sterile distilled water. The strain was grown in nutrient broth for 24 h at 28 °C and 120 rpm; then, it was centrifuged at 3500 rpm for 10 min. The pellet was re-suspended in sterile distilled water up to the desired cell concentration. Then, plants (germinated under axenic conditions) were inoculated at day 0 and day 15 with 1×10^8 CFU of TRQ65. The control treatment (uninoculated plants) was only sprayed with sterile distilled water. Two biological replicates ($n = 15$ wheat plants) of each treatment were carried out, grown under a

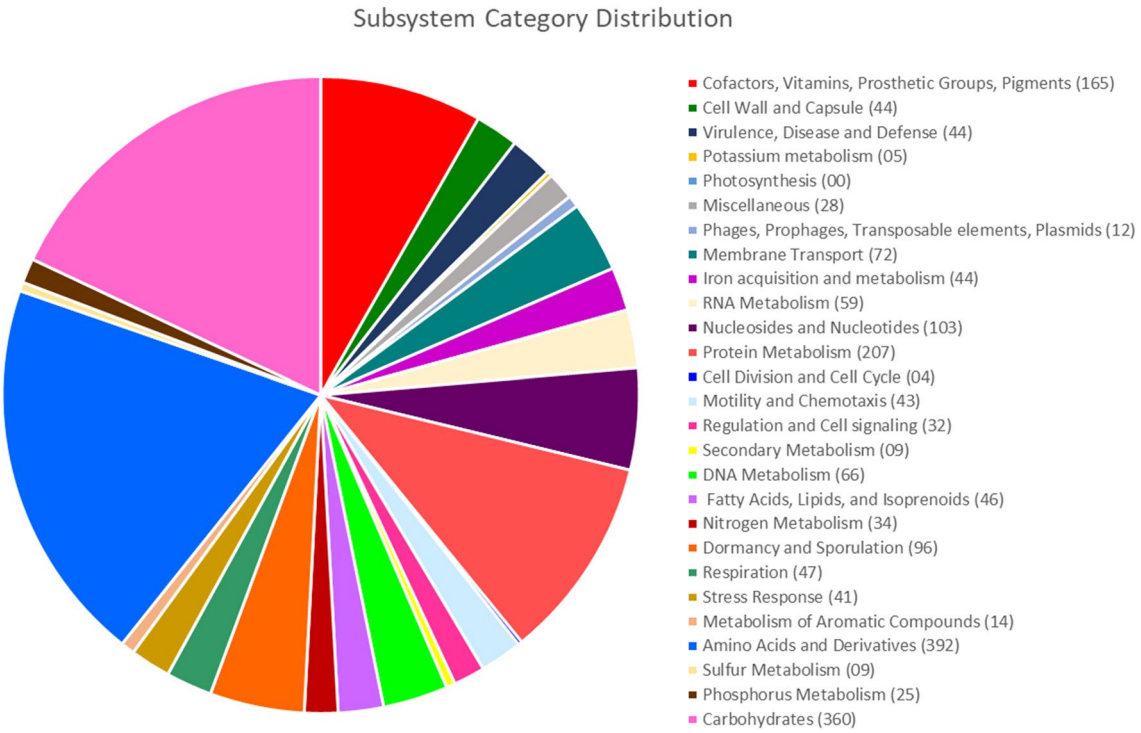

Fig. 2 Subsystem category distribution of coding DNA sequences (CDS) from strain TRQ65, generated through RASTtk pipeline. CDS: 4811, CDS in subsystems: 1419, and subsystems: 361

sterilized (5 times at 121 °C and 15 psi, for 1 h) soil–perlite (70:30) mixture. The assay was carried out in a growth chamber BJPX-A450-BIOBASE, under axenic conditions and 70% humidity, and a photoperiod 12 h light/dark (25 °C during the light, and 15 °C during dark) for 30 days. The analysis of plant biometrics was done according to Thilagar et al. (2016). The percentage of wheat growth promotion by TRQ65 was calculated by subtracting the value (cm or g) of inoculated plants minus the value (cm or g) of un-inoculated plants, and dividing by the value (cm or g) of uninoculated plants. The inoculation of strain TRQ65 to wheat plants showed a significant (Tukey–Kramer test, $p = 0.05$) increment (compared to uninoculated plants) of shoot height (26.14%), root length (36.43%), stem diameter (53.33%), stem circumference (54.34%), shoot dry weight (100%), and biovolume index (146.05%) (Table 2). These findings strongly validate the ability of strain TRQ65 to promote wheat growth, through metabolites produced by those putative genes found in its genome (Table S1) and/or novel genes that need to be studied.

In conclusion, the obtained genomic findings—and the phenotypic traits previously reported by Valenzuela-Aragon et al. (2018), Villa-Rodríguez et al. (2019), and Diaz-Rodriguez et al. (2019)—strongly confirm that strain TRQ65 belongs to *Bacillus paralicheniformis*. In addition, its genome contains genes involved in tolerance of abiotic stress conditions, biological control of phytopathogens, and plant growth promotion. Therefore, the genomic, metabolic, and ecological background observed in *Bacillus paralicheniformis* TRQ65 suggests this strain as a promising plant growth-promoting bacterium, where further analysis regarding other functional genes are required for its industrial usage as a microbial inoculant to produce wheat and other economic crops.

Table 2 Wheat growth promotion by the inoculation of *Bacillus paralicheniformis* TRQ65 (growth chamber assay)

Variable	Un-inoculated	*Bacillus paralicheniformis* TRQ65
Shoot height (cm)	18.59 ± 5.41a	23.45 ± 3.26b
Root length (cm)	7.41 ± 2.24a	10.11 ± 2.53b
Stem diameter (cm)	0.15 ± 0.04a	0.23 ± 0.04b
Stem circumference (cm)	0.46 ± 0.13a	0.71 ± 0.13b
Shoot dry weight (g)	0.05 ± 0.01a	0.10 ± 0.03b
Root dry weight (g)	0.09 ± 0.02a	0.10 ± 0.01a
Biovolume index	68.79 ± 29.96a	169.26 ± 47.90b

Means ($2 \times n = 15$) with the same letter are not significantly different, according to Tukey–Kramer test ($p = 0.05$)

Accession numbers The assembled contigs were deposited in the DDBJ/ENA/GenBank and published with the

accession number SAZD00000000. The version described in this paper is the first version of the genome sequence deposited.

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Author contributions VV: conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. RIRM: conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft. FP: conceptualization; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. GS: visualization; writing—original draft; writing—review and editing. MCO: visualization; writing—original draft; writing—review and editing. RRR: visualization; writing—original draft; writing—review and editing. SS: conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing—original draft; writing—review and editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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