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Bacillus valezensis: A New Plant Growth Promoting Rhizobacterium for Plant Growth Promotion and Inhibition of *Rhizoctonia bataticola* for the Management of Dry Root Rot of Chickpea

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ABSTRACT

Background: Chickpea production is threatened by dry root rot disease in recent years. The disease is caused by soil borne fungus *Rhizoctonia bataticola* (Taub) Butler with its pycnidial stage *Macrophomina phaseolina* (Tassi) Goid. *Bacillus* spp. is a rod shaped and gram negative rhizobacterium which is predominant in the soil. Hence, considering the economic importance of disease, the present investigation was carried out to repress the pathogen by using indigenous *Bacillus* spp. strains related to antagonistic potential and plant growth promoting traits.

Methods: Thirty indigenous bacterial PGPR strains were isolated from healthy rhizospheric soil samples of chickpea and their antagonistic potential was studied. Later, the potential ones were examined for plant growth promoting traits. The promising strains were identified at molecular level16S rDNA.

Result: All thirty PGPR strains of rhizospharic *Bacillus* were potentiality antagonistic against *R. bataticola* and nine strains showed more than 50 per cent inhibition of the pathogen. Out of nine strains, four strains recorded more growth promoting traits and they were identified at molecular level as *Bacillus cereus, Bacillus velezensis*, *Bacillus subtilis* and *Bacillus subtilis* sub sp. *subtilis. Bacillus valezensis* is a new report on rhizosphaeric PGPR against *R. bataticola* in chickpea.

Key words: Antagonistic potential, *Bacillus valezensis*, Chickpea, PGPR, *Rhizoctonia bataticola.*

INTRODUCTION

Dry root rot in chickpea caused by *Rhizoctoniabataticola* is an economically important disease and results in more yield losses wherever the crop is grown across the globe as well as in India in recent years. All the states wherever chickpea is grown are affected by the disease and it is particularly severe in Karnataka, Maharashtra, Gujarat, Tamil Nadu, Madhya Pradesh, Tellangana and Andhra Pradesh. The disease is more prevalent between flowering and podding stages and causes yield loss up to 100 per cent in susceptible varieties of chickpea under favorable conditions (Gupta and Sharma, 2015).

At present, the disease management strategy relies heavily on the use of fungicides, which are not eco-friendly and affordable to many farmers under organic farming of chickpea. Additionally, the use of plant growth-promoting rhizobacteria (PGPR) is potentially advantageous for improving crop productivity, food quality and security in more sustainable and eco-friendly manner (Etesami, 2020).

Hence, keeping the above facts in view, use of plant growth promoting rhizobacteria. *Bacillus* spp. is a rod-shaped, gram positive bacterium, frequently found in soil and reported to produce endospores to resist dry environments and high temperatures. The aim of present investigation was to evaluate the antagonistic potential, plant growth promoting traits of indigenous *Bacillus* sp. strains against *Rhizoctonia bataticola* causing dry root rot of chickpea and to identify them at molecular level using 16S rDNA.

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MATERIALS AND METHODS

Isolationand maintenance of the pathogen

Plants showing typical dry root rot symptoms were collected from chickpea fields of UAS, Raichur during *rabi* 2020-21 cropping season and the pathogen was isolated from the infected portions and cultured on potato dextrose agar (PDA) medium using hyphal tip isolation method. Pure cultures of the pathogen *R. bataticola* were maintained on PDA slants.

Collection and isolation of native *Bacillus* PGPR strains

Soil samples from healthy chickpea rhizosphere were collected during *rabi*, 2020 for isolation PGPR. Nineteen plant growth promoting rhizobacteria, *Bacillus* sp. strains were

obtained by serial dilution technique using Hichrome *Bacillus* agar medium (Waksman, 1922) from different regions of Karnataka (Table 1). The *Bacillus* sp. strains were kept under observation daily for the appearance of green colonies.

Antagonistic potential of PGPR strains against *R. bataticola*

Thirty PGPR strains of *Bacillus* sp. were screened for antagonistic potential against *R. bataticola* by dual culture technique (Xu and Kim, 2014). The degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. The per cent inhibition over the control was calculated by using the formula (Vincent, 1927).

$$I = \frac{C - T}{C} \times 100$$

Where;
I = Per cent inhibition of mycelium.
C = Growth of fungal mycelium in control.
T = Growth of fungal mycelium in treatment.

The efficient PGPR strains of indigenous *Bacillus* sp. which showed more than 50 per cent mycelial inhibition of pathogen were selected to study plant growth promoting traits.

Plant growth promoting traits of potential PGPR

Indole acetic acid (IAA) production

It was measured by the method described by Patten and Glick (2002) and the detection of IAA was determined by the development of pink color.

Ammonium production

The test was carried out as explained by Cappuccino and Sherman (1992). Development of brown to yellow colour indicated positive test for ammonium production.

Phosphate solubilization

The phosphate solubilization study was conducted as per protocol given by Nautiyal (1999). Test PGPR strain was stabbed on plate using sterile cork borer. The observation on halo zone was measured after 3 days of incubation at 28°C.

HCN production

Production of HCN was determined in slants containing NA supplemented with 4.4 g/l of glycine (Lorck, 1948) and the rhizobacteria were streaked and plates were inverted. Later, a piece of Whatman filter paper no. 1 impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with parafilm and incubated for and incubated at 28-30°C for 96 h. Change in colour of the filter paper from orange to brown was considered as production of HCN. Change in colour of inserted filter paper to brown or reddish-brown was recorded as positive reaction.

Molecular characterization of highly potential PGPR

The rhizospheric PGPR which showed higher plant growth promoting traits were further selected for molecular characterization. The DNA isolation of four efficient strains was carried out by using standard CTAB method. The universal primers 16s rDNA F (GAG-TTT-GAT-CCT-GGC-TCA) and 16s rDNA R (AGA-AAG-GAG-GTG-ATC-CAG) were synthesized at Eurofins, Bangalore, India for characterization of isolates. Amplification was conducted in a thermal cycler (Veriti, Applied Biosystems, Singapore) and the PCR programming was done as per Yugander *et al*. (2017). Initial denaturation was set for 1 min at 94°C for 1 cycle. Whereas, denaturation time was set for 1 min at 96°C, annealing for 1.5 min at 58°C and extension for 1.5 min at 72°C for 35 cycles. The final extension was set for 8 min at 72°C for 1 cycle.

RESULTS AND DISCUSSION

Isolation of pathogen

The results indicated that, the white mycelial growth of pathogen was observed within 3 to 4 days. Later, it turned to black colour showing sclerotial bodies after 8-10 days. The mycelium was brown in colour and branching was right angled under microscope showing specific character of the pathogen.

Isolation and maintenance of native PGPR strains of *Bacillus* sp.

Thirty strains of *Bacillus* sp.were successfully isolated by using Hichrome Bacillus agar medium (Bharose *et al*., 2017) and strains were designated as SBPGPM- 1to SBPGPM-30 (Table 2).

Antagonistic potential of rhizospheric PGPR strains against *R. bataticola*

The per cent inhibition of mycelial growth of pathogen varied greatly (2.59-83.89 mm) among the thirty isolates. Among them, nine strains*viz*., SBPGPM-4, SBPGPM-7, SBPGPM-9, SBPGPM-15, SBPGPM-19, SBPGPM-21, SBPGPM-23, SBPGPM-28 and SBPGPM-30 recorded more than 50 per cent inhibition of pathogen (Table 1 and Fig 1). However, the minimum mycelial inhibition was observed in SBPGPM-22 with per cent inhibition of 2.59 (Table 2, Fig 1). Similarly, Pandey *et al*. (2016) studied the antagonistic action of fluorescent *Pseudomonas* which was isolated from native soil (Raipur), tested against three different isolates of *F. oxysporum* f. sp. *ciceris* by dual culture technique. The fluorescent *Pseudomonas* inhibited the growth of pathogen isolates such as *F. oxysporum* f. sp. *ciceris* 1, *F. oxysporum* f. sp. *ciceris* 2 and *F. oxysporum* f. sp. *ciceris* to the extent of 46.70, 46.83 and 46.03 per cent, respectively. Likewise, *B. subtilis* strain PRBS-1 and AP-3 inhibited five soybean seed pathogenic fungi, *viz*., *Rhizoctonia solani*, *C. truncatum*, *S. sclerotium*, *M. phaseolina* and *Phomopsis* spp. under *in vitro* conditions (Araujo *et al*., 2005). In the current study also the rhizospheric PGPR strains able to inhibit the *R. bataticola* which indicated that rhizospheric bacteria can inhibit *R. bataticola*.

Plant growth promoting traits of potential PGPR strains

Plant growth promoting microorganisms significantly ameliorate plant growth by a number of mechanisms including increase of uptake of essential nutrients such as phosphate, ammonia and nitrogen, producing phytohormones such as indole-3-acetic acid and gibberellins. Hence, nine potential rhizospheric bacterial PGPMs, which were efficient in inhibition of pathogen in the dual culture assay were further screened for the production of plant growth promoting traits like IAA production, ammonia production, phosphate solubilization and HCN production (Table 3).

IAA production

Indole-3-acetic acid is the most common, naturally occurring plant hormone. IAA influences the process of forming plant tissues, namely growth, division and cell differentiation and protein synthesis. A diverse genus of microbes can augment significant amount of IAA. That being the case, in the present study,nine strains such as SBPGPM-4, SBPGPM-7, SBPGPM-9, SBPGPM-15, SBPGPM-19, SBPGPM-21, SBPGPM-23, SBPGPM-28 and SBPGPM-30exhibited pink color which indicated the production of indole-3-acetic acid as detected by the Salkowaski's reagent (Table 3 and Fig 2a). The results indicated that PGPR strains have the ability to induce plant growth promotion of chickpea through the synthesis of IAA which helped in plant cell elongation, proliferation and also indirectly supported the growth of root and shoot. The present results are supported by Reetha *et al*. (2014) who undertook a study for isolation of *P. fluorescens* and *B. subtilis* from rhizosphere of onion and analysis of these bacteria for *in vitro* IAA acetic acid production. The results indicated that the two tested PGPR exhibited a pink to red color with a little variation in intensity.

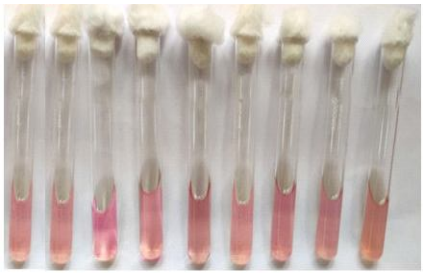
Ammonium production

Ammonium plays a key role in the plant growth by providing the nitrogen to plants.All nine strains (SBPGPM–4,

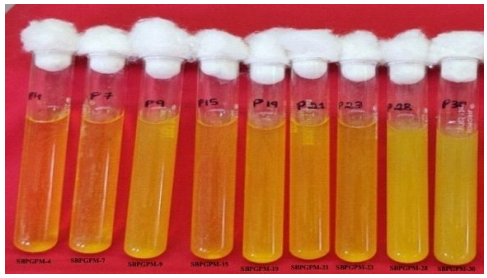
Table 1: Source and designation of chickpea rhizospheric bacterial PGPM isolates.

District	Village	No. of bacterial isolates obtained	Isolate code
Dharwad	Garag	1	SBPGPM-1
	Thadakoda	1	SBPGPM-2
	Narendra	1	SBPGPM-3
Gadag	Gajendrigad	2	SBPGPM-4
			SBPGPM-5
Haveri	Chandapura	3	SBPGPM-6
			SBPGPM-7
			SBPGPM-8
Kalaburagi	ZARS, Kalaburagi	1	SBPGPM-9
Yadgiri	Bheemarayanagudi	2	SBPGPM-10
			SBPGPM-11
Bidar	Basavakalyan	1	SBPGPM-12
	Dubalgundi	1	SBPGPM-13
	Hudugi	1	SBPGPM-14
Vijayapura	Hittinhalli	1	SBPGPM-15
Bagalkot	Bagalkot	2	SBPGPM-16
			SBPGPM-17
Ballari	Ballari	2	SBPGPM-18
			SBPGPM-19
Koppal	Kushtagi	4	SBPGPM-20
			SBPGPM-21
			SBPGPM-22
			SBPGPM-23
	Ganganal	1	SBPGPM-24
	Hiresindhogi	1	SBPGPM-25
	Yalburga	1	SBPGPM-26
Raichur	New area		SBPGPM-27
	(UAS campus)	2	SBPGPM-28
	Askihal	1	SBPGPM-29
	Rampur	1	SBPGPM-30

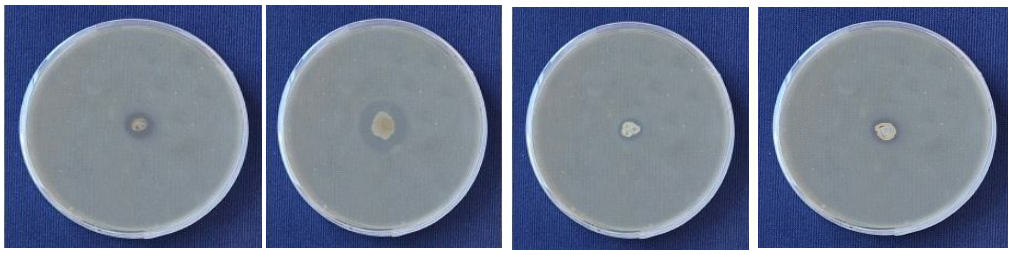
Fig 1: Inhibition of *R. bataticola* by rhizospheric bacterial PGPMs in dual culture assay.



a. IAA production



b. Ammonium production



c. Phosphate solubilization

d. HCN production

Fig 2: Plant growth promoting traits exhibited by respective PGPMs.

SBPGPM-7, SBPGPM-9, SBPGPM- 15, SBPGPM-19, SBPGPM-21, SBPGPM- 23, SBPGPM-28 and SBPGPM-30) were found positive for production of ammonium by exhibiting yellow color (Table 3 and Fig 2b). The results also indicated that the PGPMs which could produce the ammonia can supply the crops with a sufficient amount of ammonium required for root and shoot elongation and consequently promote plant growth.

Table 2: Antagonistic potential of rhizospheric bacterial PGPMs against *R. bataticola* in dual culture assay.

Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
SBPGPM- 1	59.67	33.70 (35.48)
SBPGPM- 2	73.17	18.70 (25.62)
SBPGPM- 3	64.00	28.89 (32.51)
SBPGPM- 4	36.67	59.26 (50.33)
SBPGPM- 5	70.00	22.22 (28.12)
SBPGPM- 6	52.67	41.48 (40.09)
SBPGPM- 7	37.17	58.70 (50.01)
SBPGPM- 8	64.83	27.96 (31.92)
SBPGPM- 9	36.50	59.44 (50.44)
SBPGPM- 10	84.50	6.11 (14.31)
SBPGPM- 11	84.33	6.30 (14.53)
SBPGPM- 12	79.00	12.22 (20.46)
SBPGPM- 13	79.83	11.30 (19.64)
SBPGPM- 14	54.83	39.07 (38.68)
SBPGPM- 15	38.83	56.85 (48.93)
SBPGPM- 16	70.67	21.48 (27.61)
SBPGPM- 17	63.50	29.44 (32.86)
SBPGPM- 18	65.33	27.41 (31.56)
SBPGPM- 19	42.67	52.59 (46.48)
SBPGPM- 20	71.27	21.26 (27.45)
SBPGPM- 21	14.97	83.89 (66.33)
SBPGPM- 22	88.00	2.59 (9.26)
SBPGPM- 23	31.33	67.41 (55.18)
SBPGPM- 24	65.33	28.33 (32.16)
SBPGPM- 25	68.00	26.30 (30.85)
SBPGPM- 26	65.23	29.44 (32.86)
SBPGPM- 27	70.00	22.59 (28.38)
SBPGPM- 28	44.97	50.93 (45.52)
SBPGPM- 29	84.50	9.26 (17.71)
SBPGPM- 30	22.67	76.30 (60.86)
Control	90.00	0.00 (0.00)
S. Em ±	-	1.06
CD at 1%	-	3.97

There is a supporting report given by Hassan (2017) that the rhizobacteria such as *B. cereus* and *B. subtilis* and fungal PGPMs, *P. chrysogenum* and *P. crustosum* also produced indole acetic acid, ammonium and phosphatein *Teucrium polium.*

Phosphate solubilization

Phosphorus is one of the macronutrients required for plant growth promotion. In most cases, phosphorus is present in the soil as insoluble inorganic forms. But, different rhizospheric and endophytic PGPM strains have the efficacy to convert it from an unavailable to available source for plant uptake. In the present study, the phosphate solubilizing activity for all nine isolates was assessed on Pikoviskaya medium supplemented with tri-calcium phosphate as an inorganic phosphate source. Out of nine, five isolates such as SBPGPM-9, SBPGPM-19, SBPGPM-21, SBPGPM- 23, SBPGPM-28 were positive for the reaction (Table 3 and Fig 2c).

HCN production

HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens. Among the nine strains tested, five strains *viz*., SBPGPM-19, SBPGPM-21, SBPGPM-23, SBPGPM-28, SBPGPM-30 produced light reddish brown color on the filter paper which indicated the production of HCN (Table 3 and Fig 2d).Similar type of results was obtained with Ramyabharathi and Raguchander (2014), who reported that, *B. subtilis* was found to be positive for hydrogencyanide production.

There is a previous evidence similar to present investigation in which one hundred and fifty strains (endophytic and rhizospheric PGPMs) isolated from *Canola* were characterized for plant growth promoting traits. Among them, hundred isolates produced indole-3-acetic acid, seventeen isolates solubilized phosphate, forty four isolates produced siderophores, thirty four produced 1-aminocyclopropane-1-carboxylate deaminase and five produced hydrocyanic acid (Etesami *et al*., 2014). In the

Table 3: Screening of potential PGPMs for plant growth promoting traits.

Isolate code	IAA production	Siderophore production	Ammonium production	Phosphate solubilization	HCN production
SBPGPM-4	+	-	+	-	-
SBPGPM-7	+	-	+	-	-
SBPGPM-9	+	-	+	+	-
SBPGPM-15	+	-	+	-	-
SBPGPM-19	+	-	+	+	+
SBPGPM-21	+	-	+	+	+
SBPGPM-23	+	-	+	+	+
SBPGPM-28	+	-	+	+	+
SBPGPM-30	+	-	+	-	+

'+' - Indicates positive reaction for the test'-'- Indicates negative reaction for the test.

present investigation also the native rhizospheric PGPR strains could produce similar PGP traits.

It is clear from the present investigation that out of nine potential PGPR strains, only four PGPR strains namely SBPGPM-19, SBPGPM-21, SBPGPM- 23 and SBPGPM-28 successful in exhibiting more PGP traits tested. Hence, all the four native PGPRstrains were further carried for molecular characterization.

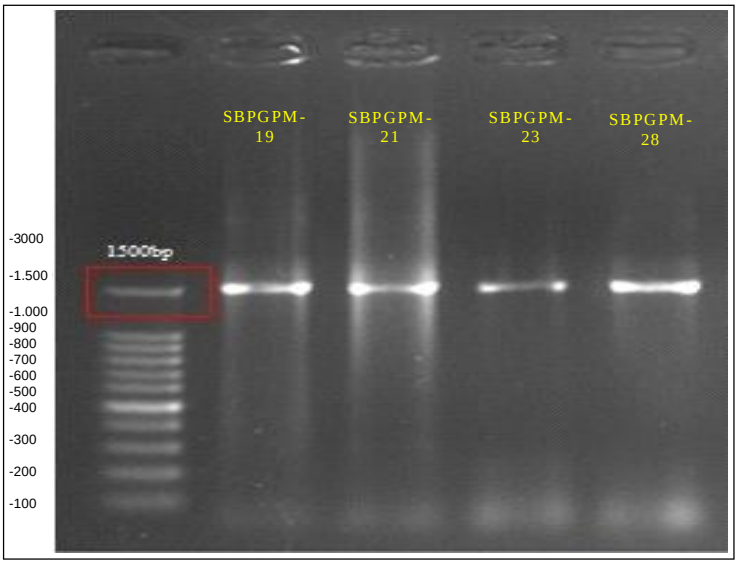


Fig 3: Gel picture showing amplified products of rhizospheric bacterial PGPM isolates.

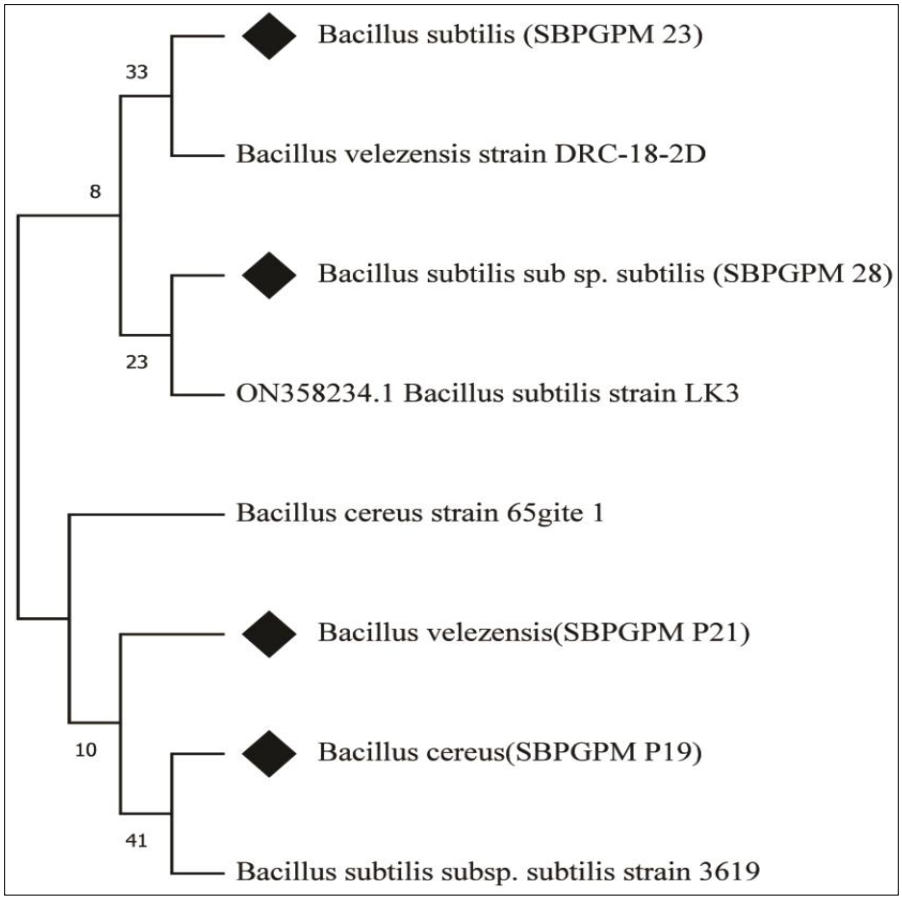


Fig 4: Phylogenetic tree depicting diversity among the rhizospheric bacterial PGPM isolates.

Table 4: Molecular characterization of rhizospheric bacterial PGPMs by 16S rDNA sequencing.

Isolate code	Strain	Accession number	Per cent identity	Reference organism
SBPGPM-19	P 19	ON567448	100%	*Bacillus cereus*
SBPGPM-21	P 21	ON568504	100%	*Bacillus velezensis*
SBPGPM -23	P 23	ON566236	100%	*Bacillus subtilis*
SBPGPM-28	P 28	ON566124	99.34%	*Bacillus subtilis* subsp. *subtilis*

Molecular characterization of highly potential PGPR strains

Four highly potential rhizospheric bacterial strains such as SBPGPM-19, SBPGPM-21 SBPGPM- 23 and SBPGPM- 28 were amplified for 16S rRNA gene (Table 4, Fig 3). The results indicated the size of PCR amplified products of all four strains was 1500 bp. Four strains such as SBPGPM-19, SBPGPM-21 SBPGPM-23 and SBPGPM-28 were identified as *B. cereus* (Acc. No. ON567448), *B. velezensis* (ON568504), *B. subtilis* (ON566236), *B.subtilis*sub sp. *subtilis* (ON566124), respectively and the accession numbers were deposited in genebank. Further, diversity analysis among them was studied by phylogenetic analysis. The phylogeny results recorded homology with *B. cereus* (100 %), *Bvelezensis* (100%), *B. subtilis* (100%), *B. subtilis* sub sp. *subtilis* (99.34%) (Fig 4). Among four highly potential rhizosphaeric PGPR, *Bacillus velezensis* is new report for antagonistic potential and plant growth promotion in case of chickpea crop.

CONCLUSION

The present investigation put forward about the exploitation of plant growth promoting microorganisms for the pathogen suppression. In another terms, a healthy plant with its associated microorganisms can fight back against pathogens. As an evidence, in the current study, *Bacillus valezensis*, isolated from healthy chickpea rhizosphere could inhibit *Rhizoctonia bataticola* which causes major threat to its host. Additionally, it could exhibit plant growthpromoting activities. It is the first report that *Bacillus valezensis* could inhibit the pathogen.

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Conflict of interest: None.

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