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Direct and indirect plant growth-promoting abilities of *Bacillus* species on chickpea, isolated from compost and rhizosphere soils

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Abstract A study was carried out to test the effect of direct and indirect plant growth-promoting traits of bacteria, isolated from compost and rhizosphere soils, on chickpea. A total of 74 bacteria were isolated from herbal vermicomposts and rhizosphere soils of chickpea and screened for their antagonistic potential against soil-borne fungal pathogens of chickpea. Of which, four bacterial isolates (VBI-4, VBI-19, VBI-23, and SBI-23) were found to be promising in both dual culture and metabolite production assays. These isolates were identified as *Bacillus* species by 16S ribosomal DNA (rDNA) sequence analysis. Under in vitro conditions, all the isolates were found to produce protease, cellulase, β-1,3-glucanase, siderophore, indole acetic acid, lipase (except VBI-19), and hydrocyanic acid (except VBI-23 and SBI-23). All the isolates were tolerant to fungicides such as bavistin, captan, benlate, ridomil (only VBI-23 and SBI-23), and thiram (only VBI-4 and VBI-19) at field application rates. The isolates were also found to tolerate NaCl concentration of up to 8 % (VBI-23 up to 10 %), temperature range of 20 to 40 °C, and a pH range of 7 to 11 (SBI-23 up to only 9). When the isolates were evaluated for their plant growth promotion (PGP) ability under greenhouse and field conditions on chickpea, all the isolates were able to increase growth parameters including nodule number, plant growth, and yield parameters when compared to uninoculated control. The isolates also increased the soil mineral properties including total N, available P, organic carbon (OC) %, microbial biomass C, and dehydrogenase activity in rhizosphere, at both flowering and harvest stages over the uninoculated control plots. All the isolates were found to colonize chickpea roots when observed under scanning electron microscope. This investigation indicated the PGP potential of selected bacteria in chickpea cultivation.

Keywords Plant growth promotion · Bacteria · Chickpea · Colonization · *Bacillus* · SEM analysis

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Introduction

Chickpea (*Cicer arietinum* L.) is the second most cultivated pulse crop in the world, after common bean with an annual production of 13.8 million tons (FAOSTAT 2014). The favorable conditions for growing chickpea are low temperatures, less rainfall, and soils with neutral pH. Chickpea is relatively drought tolerant when compared with other pulses. However, it is sensitive to high moisture and high temperatures (Clarke and Siddique 2004). Chickpea crop is affected by the number of diseases at various stages of its growth. Of which, diseases caused by fungi are important because they are easily propagated and are known to cause huge losses in productivity. The crop losses due to fungal

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pathogens can be sometimes up to 100 % (Akhtar and Siddiqui 2010). Usually, soil-borne fungal pathogens are controlled by chemicals; however, this practice also leads to other environmental and health concerns. Approximately 2.5 million tons of pesticides are used annually worldwide which in return are accumulating in to the environment (Rao et al. 2015). In order to avoid this problem, biological control methods are followed where a group of microbes are used to control phytopathogens. Bacteria which are present in soil and help plants to promote their growth and development are called plant growth-promoting rhizobacteria (PGPR). PGPR enhances plant growth by two ways, either directly by producing phytohormones such as indole acetic acid (IAA) and siderophores (making Fe available for growth) or indirectly by producing lytic enzymes, antibiotic compounds, and volatile compounds such as hydrocyanic acid (HCN) and chitinase and control soil-borne pathogens. PGPR such as *Bacillus*, *Azotobacter*, *Pseudomonads*, *Burkholderia*, and *Enterobacter* are reported in promoting plant growth and yield by both direct as well as indirect means (El-Tarabily et al. 2009). PGPR are usually isolated from compost and rhizosphere of economically important crops. The main objectives of the present study were to isolate bacteria from herbal vermicomposts and rhizosphere soils of chickpea, to screen for their antagonistic potential against soil-borne fungal pathogens of chickpea, and to test the direct and indirect PGP abilities on chickpea, under in vitro, greenhouse and field conditions.

Materials and methods

Isolation of bacteria

Ten grams of either herbal vermicompost (*Annona squamosa*, *Gliricidia sepium*, *Jatropha curcas*, *Azadirachta indica*, and *Parthenium hysterophorus*) or chickpea rhizosphere soil (collected at the depth of 0 15 cm with the help of soil core randomly in the chickpea fields) was added in 90 ml of physiological saline and allowed for shaking on an orbital shaker for 60 min. At the end of incubation, the samples were serially diluted and plated on Luria Bertaini (LB) agar (HiMedia laboratories, Mumbai, India) and further incubated at 28 ±2 °C for 24 h. Individual colonies were isolated and stored on LB agar slants at 4 °C for further studies.

Screening bacteria against fungal pathogens of chickpea

The fungal pathogens of chickpea such as *Sclerotium rolfsii*, *Rhizoctonia bataticola* (three strains such as Rb-6, Rb-24, and Rb-115), and *Fusarium oxysporum* f. sp *ciceri* (FOC) were acquired from Legumes Pathology division of International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru. The isolated bacteria were screened for their antagonistic potential against selected fungal pathogens of chickpea by dual culture assay as per the protocols of Gopalakrishnan et al. (2011), and the zone of inhibition was measured.

Metabolite production assay was carried out by growing the selected bacterial isolates in LB broth at 28 °C for 3 days. At the end of incubation, the culture filtrates were collected by centrifugation at 10,000×*g* for 20 min. The pH of the culture filtrates was adjusted to 3 and partitioned against equal volumes of ethyl acetate (EtOAc). The resultant organic and aqueous fractions were collected and evaporated completely on rotary evaporator (BUCHI V-850, Switzerland), and the final remnants were collected in methanol and tested for their potential to inhibit fungal test pathogens. For this assay, potato dextrose agar (PDA) plates were amended with 0.5 % test samples whereas control plates contained 0.5 % methanol. A fungal disk was kept at the center and incubated at 28 ±2 °C for 5 days. At the end of incubation, fungal inhibition was measured and compared with control. For both dual culture and metabolite production assays, fungal inhibition was recorded on a scale of 0, 1, 2, 3, and 4 as no inhibition, slight inhibition, moderate inhibition, good inhibition, and excellent inhibition, respectively.

Evaluation of bacteria for their biochemical and physiological traits

The selected bacterial isolates were evaluated for their biochemical traits such as production of siderophore, lipase, protease, cellulase, HCN, IAA, and β-1,3-glucanase as per the protocols of Schwyn and Neilands (1987), Bhattacharya et al. (2009), Bhattacharya et al. (2009), Hendricks et al. (1995), Lorck (1948), Patten and Glick (2002), and Singh et al. (1999), respectively. Observations for production of siderophore, lipase, and protease were recorded on a 0–4 rating scale depending on the diameter of the halo zone formed around the culture. Observations of HCN were recorded on a 0–3 rating scale based on the intensity of the reddish brown color.

The selected bacteria were also tested for their physiological traits such as tolerance to salinity, pH, temperature, and fungicides (tolerant/sensitive) according to the protocols developed by Gopalakrishnan et al. (2014).

Molecular identification of selected bacterial isolates

The selected bacteria were sent to Macrogen Inc., Seoul, Korea, for identification based on their 16S ribosomal DNA (rDNA) analysis. Macrogen amplified the 16S rDNA gene using universal bacterial primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The obtained sequences were compared with similar sequences retrieved from GenBank using the BLAST and aligned using the Clustal W software, and the dendrogram was constructed by neighbor-joining method (Altschul et al. 1990; Thompson et al. 1997; Saitou and Nei 1987). Bootstrap analysis was performed using the MEGA version 4 program for estimation of the statistical stability of the branches in cluster with 1000 replications.

Evaluation of the bacteria for their PGP potential on chickpea under greenhouse and field conditions

For greenhouse studies, pot mixture (black soil, sand, and farm yard manure; 3:2:2) were filled in 8″ pots. Chickpea seeds (variety ICCV 2) were sterilized (with 2.5 % sodium hypochlorite and rinsed several times with sterile water) and soaked in selected bacterial cultures for 50 min (10^8 CFU ml^{-1}; grown in LB broth separately). Five treatments (VBI-4, VBI-19, VBI-23, SBI-23, and control) were kept, and the experiment was carried out with six replications. Six seeds were sown in the pots, but three plants were maintained after germination. The bacterial cultures (10^8 CFU ml^{-1}) were applied once in 2 weeks until flowering stage as booster dose. Irrigation and pest management were done as and when required. Growth parameters such as nodule number, nodule dry weight, plant height, leaf area, leaf weight, shoot weight, root length, and root volume were recorded at 30 days after sowing (DAS), and stem weight, pod weight, and pod number were recorded at harvesting stage.

The PGP potential of the bacterial isolates was also tested on chickpea under field conditions. The experiment was carried out in 2013–2014 cropping seasons (post-rainy) at ICRISAT Patancheru, Telangana, India. The soil in experimental field was Vertisol type. The rhizosphere soil contains 0.56 % organic C, 642 ppm total N, and 9.03 ppm available P. Randomized complete block design was used as experimental layout, while the plot size was maintained at 4 m × 3 ridges. Chickpea seeds (variety ICCV 2) were surface sterilized and soaked in bacterial cultures as described earlier and sown on 2 November 2013 at a row-to-row spacing of 60 cm and a plant-to-plant spacing of 10 cm. The booster doses of bacterial isolates were given every 2 weeks to the treatment plots until flowering stage. The control plots contained no bacterial culture. Irrigation and weeding were done as required. Plant growth parameters including nodule number, leaf area, leaf weight, pod number, shoot weight, and plant height were taken at 60 DAS and compared with control. The crop was harvested manually on 4 February 2014, and observations including stover yield and grain yield were noted.

Rhizosphere soil samples (0–15 cm) were collected at flowering and harvest and analyzed for total N, available P, and organic carbon (OC) % as per the protocols of Novozamsky et al. (1983), Olsen and Sommers (1982), and Nelson and Sommers (1982), respectively, while soil microbial biomass C and dehydrogenase activity were estimated as per the protocols of Anderson and Domsch (1989) and Casida (1977), respectively.

Colonization capability of selected bacterial isolates on chickpea roots

Chickpea roots were examined for colonization by bacteria using scanning electron microscope (SEM) analysis as per the protocols of Bozzola and Russell (1999). Chickpea (ICCV 2) root tips were collected and processed according to the procedure described by Gopalakrishnan et al. (2014). The samples were examined under scanning electron microscope (JOEL-JSM 5600) as per the standardized protocols at RUSKA Lab, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India, and observed for colonization of bacteria on the roots of chickpea.

Statistical analysis

Data were analyzed by using analysis of variance (ANOVA; Gen Stat 10.1 version 2007) technique considering isolates and replication. Isolate means were tested for significance and compared using Fisher's protected least significant difference (LSD).

Results and discussion

Isolation and screening of bacteria for their antagonistic potential against fungal pathogens of chickpea

In the present study, a total of 74 bacteria were isolated from chickpea rhizosphere and five herbal vermicompost and screened for their antagonistic potential against the selected important fungal pathogens of chickpea by dual culture and metabolite production assays. Of the 74 bacterial isolates, four namely VBI-4 (from *A. squamosa* vermicompost), VBI-19 (from *G. sepium* vermicompost), VBI-23 (from *J. curcas* vermicompost), and SBI-23 (from chickpea rhizosphere soil) were selected based on their broad-spectrum antagonistic potentials for further studies. The selected four isolates inhibited all the pathogens tested in both dual culture and metabolite production assays. Of the four selected isolates, VBI-23 was found to be more effective in both the assays when compared to the other isolates (Table 1). Among the four selected bacterial isolates, three (VBI-4, VBI-19, and VBI-23) were isolated from vermicompost. Microbes isolated from compost are reported to have potential in controlling phytopathogens. For example, *Aspergillus* spp. isolated from vermicompost was found effective in controlling *Fusarium oxysporum* f. sp. *melonis* causing wilt in melon (Suarez-Estrella et al. 2007); *Penicillium citrinum* isolated from vermicompost was found to inhibit *Botrytis cinerea* causing botrytis gray mold in chickpea (Sreevidya et al. 2015). Bacteria including *Pseudomonas*, *Serratia*, *Enterobacter*, and *Bacillus* isolated from compost were also proved to inhibit phytopathogens of turfgrass (Boulter et al. 2002).

Biochemical and physiological traits of selected bacterial isolates

In the present study, all the selected bacterial isolates were found to produce extracellular enzymes including protease, lipase (except VBI-19), cellulase, β-1,3-glucanase, and PGP substances such as siderophores, IAA, and volatile compounds such as HCN (except VBI-23 and SBI-23) (Fig. 1). The ability to produce extracellular enzymes by PGP bacteria helps in controlling the soil-borne pathogens by acting on their cell walls (Ellis et al. 2000), thereby indirectly functions as PGP. Phytohormones are plant growth regulators, which influence the growth of plants. Auxins (such as IAA) are one of the phytohormones regulating cell differentiation, root elongation, fruit formation, and abscission control (Khamna et al. 2009). Siderophores are the low molecular weight Fe-binding compounds, which binds Fe^{3+} and convert it to readily absorbable form (Gray and Smith 2005), which can be used by the plants. HCN is a volatile antibiotic that helps in disease suppression (Siddiqui 2006). HCN produced by *Pseudomonas fluorescens* strain CHA0 was reported to suppress black root rot disease in tobacco (Keel et al. 1989).

When the selected four bacterial isolates were tested for their tolerance to salinity, pH, and temperature, all the isolates tolerated NaCl concentration up to 8 % (VBI-23 tolerated up to 10 %), temperatures 20–40 °C, and pH 7–11 (except SBI-23 which tolerated up to pH 9). The selected bacteria were also tested for fungicide tolerance at field application levels, as, if the bacteria were compatible with fungicides, the concentration of fungicide required for field application can be minimized. In the present study, all the selected isolates were tolerant to

Table 1 Effect of bacterial isolates for their antagonistic potential against fungal pathogens of chickpea

Isolate	Dual culture assay					Metabolite production assay				
	RB-6	RB-24	RB-115	FOC	*S. rolfsii*	RB-6	RB-24	RB-115	FOC	*S. rolfsii*
VBI-4	2.7	2.7	1.0	1.0	3.0	3.0	3.0	3.0	1.0	1.0
VBI-19	1.3	2.3	1.0	1.0	3.0	3.0	3.0	3.0	1.0	1.0
VBI-23	1.3	3.0	1.0	2.0	3.0	3.0	3.0	3.0	2.0	2.0
SBI-23	1.0	1.0	1.7	1.0	3.0	2.0	3.0	2.0	1.0	1.0
Control	0	0	0	0	0	0	0	0	0	0
LSD (5 %)	0.75	0.75	0	0	0	0	0	0	0	0

RB-6, *RB-24*, and *RB-115* three strains of *Rhizoctonia bataticola*, *FOC Fusarium oxysporum* f. sp. *ciceri*, *S. rolfsii Sclerotium rolfsii*, rating scale *0* no inhibition, *1* slight inhibition, *2* moderate inhibition, *3* good inhibition, *4* excellent inhibition, *LSD* least significant difference

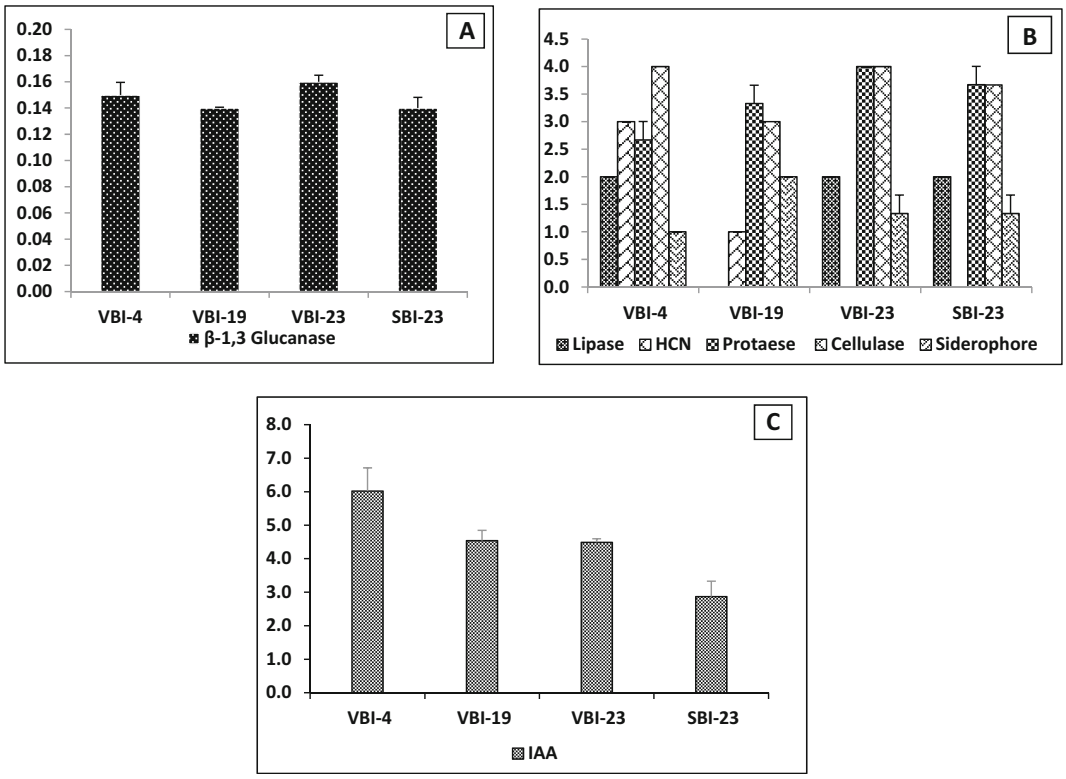

Fig. 1 Production of β-1, 3-glucanase (**a**), lipase, HCN, protease, cellulase, and siderophore (**b**), and IAA (**c**) by four bacterial isolates

fungicides bavistin, captan, and benlate, while VBI-23 and SBI-23 were also found tolerant to ridomil and VBI-19 and VBI-4 were tolerant to thiram at field application levels (Table 2). The ability of bacteria to adapt extreme conditions such as higher temperatures, alkaline, or acidic pH and higher saline conditions makes them better survive in soils with acidic or alkaline pH and extreme climatic conditions. These traits were reported to help the bacterial isolates to compete and colonize in rhizosphere when inoculated in to soil (Habe and Uesughi 2000).

Molecular identification of selected bacterial isolates

When the sequences of the selected bacterial isolates were analyzed, the results revealed that all isolates matched (100 %) with *Bacillus* spp. (Fig. 2). The sequences of 16S rDNA of VBI-4 (1492 bp), VBI-19 (1526 bp), VBI-23 (1494 bp), and SBI-23 (1490 bp) were submitted to GenBank, and accession numbers KM250376, KM250377, KM250378, and KM250375, respectively, were obtained.

Table 2 Effect of temperature, pH, salinity, and fungicides on the growth of selected four bacterial isolates

Isolate	Temperature	pH	NaCl %	Fungicide tolerance/sensitive at field application levels				
				Bavistin (2500 ppm)	Thiram (3000 ppm)	Benlate (4000 ppm)	Captan (3000 ppm)	Ridomil (3000 ppm)
VBI-4	20 to 40 °C	7 to 11	Up to 8 %	Tolerant	Tolerant	Tolerant	Tolerant	Sensitive
VBI-19	20 to 40 °C	7 to 11	Up to 8 %	Tolerant	Tolerant	Tolerant	Tolerant	Sensitive
VBI-23	20 to 40 °C	7 to 11	Up to 10 %	Tolerant	Sensitive	Tolerant	Tolerant	Tolerant
SBI-23	20 to 40 °C	7 to 9	Up to 8 %	Tolerant	Sensitive	Tolerant	Tolerant	Tolerant

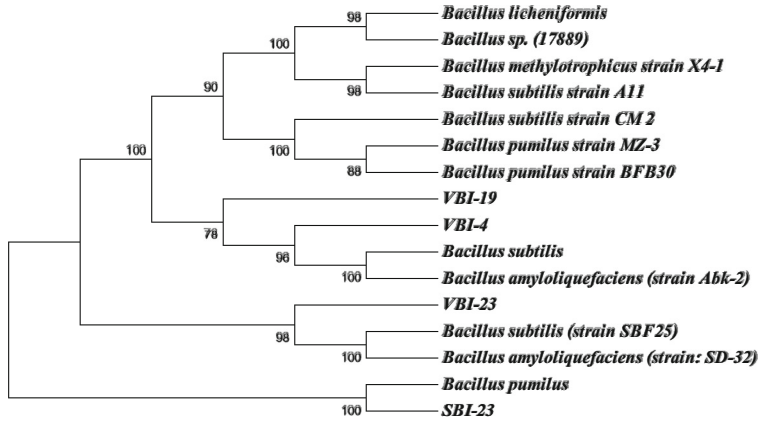

Fig. 2 Phylogenetic relationship between VBI-4, VBI-19, VBI-23, and SBI-23 representative species based on full-length 16S rDNA sequences constructed using the neighbor-joining method

Evaluation of selected PGP bacterial isolates for their PGP traits on chickpea under greenhouse and field conditions

Studies reported that PGP bacteria have the ability to increase the growth and yield of agriculturally important crops. Hence, the selected bacterial isolates were tested for their PGP ability on chickpea under greenhouse and field conditions. In the present study, under greenhouse conditions, at 30 DAS, there was an increase in the plant height (up to 17 %), nodule number (up to 6 %), nodule dry weight (up to 19 %), root length (up to 12 %), root volume (up to 11 %), leaf area (up to 22 %), leaf weight (up to 36 %) and shoot weight (up to 22 %) and at harvest, the stem weight (up to 38 %), pod weight (up to 24 %), and pod number (up to 13 %) over uninoculated control (Table 3).

Under field conditions, the selected bacterial isolates also proved efficient in promoting the agronomic properties of chickpea with an increase in the plant height (up to 9 %), nodule number (up to 15 %), leaf weight (up to 26 %), leaf area (up to 26 %), shoot weight (up to 25 %), and pod number (up to 20 %) at 60 DAS, while at crop maturity, there was an increase in total dry matter (up to 17 %), stover weight (up to 22 %), and grain yield (up to19 %) over the uninoculated control (Table 4). At 60 DAS, the bacterial isolates were also found to increase the soil mineral and biological properties including total N (up to 15 %), available P (up to 30 %), OC (up to 8 %), dehydrogenase activity (up to 20 %), and microbial biomass C (up to 15 %), and at final harvest, there was an increase in total N (up to 10 %), available P (up to 35 %), organic C (up to 7 %), dehydrogenase activity (up to 23 %), and microbial biomass C (up to 35 %) over uninoculated control plots (Fig. 3).

The increased levels of N, P, and OC show that the inoculated bacterial isolates were well established in the chickpea rhizosphere and fixed atmospheric N and also hydrolyzed the inorganic phosphates to available form. The increased levels of microbial biomass C also confirms the existence of microbes in inoculated plots. Microorganisms are known to be chemo-attracted and move toward the root exudates, allowing them to colonize and multiply both in the rhizosphere and the rhizoplane (Lugtenberg and Kamilova 2009). PGP bacteria and fungi were demonstrated to increase yield in number of crops including chickpea (Shahzad et al. 2008; Gopalakrishnan et al. 2015; Sreevidya et al. 2015), strawberry (Esitken et al. 2010), and rice (Gopalakrishnan et al. 2012).

In the present study, there was an increase in soil mineral properties such as total N; available P and organic carbon were observed by four bacterial isolates and soil biological properties such as microbial biomass carbon and dehydrogenase activities. Jannouraa et al. (2013) demonstrated close relationships between grain N and P concentrations and microbial biomass C, N, and P, thus suggesting the use of soil microbial biomass as an indicator of nutrient availability to plants. Mandal et al. (2007) reported a close relationship between the soil microbial biomass and crop yields under both greenhouse as well as field conditions. Microorganisms in soil

Table 3 Effect of the selected four bacterial isolates for their PGP and yield potential on chickpea under greenhouse conditions

Isolate	At 30 days after sowing								At crop harvest		
	Nodule number (plant^{-1})	Nodule dry weight	Leaf area (cm^{-2} plant^{-1})	Leaf weight (g plant^{-1})	Shoot weight (g plant^{-1})	Plant height (cm)	Root length (cm)	Root volume (cm^3)	Stem weight (g)	Pod weight (g)	Pod number
VBI-4	34	0.16	265	1.82	1.2	34.3	3784	11.2	2.43	3.73	18
VBI-19	34	0.15	276	1.71	1.3	35.0	3893	11.5	2.45	3.66	19
VBI-23	35	0.13	270	1.87	1.3	36.3	3697	11.1	3.31	4.66	20
SBI-23	36	0.14	335	2.21	1.5	36.0	3477	12.5	3.60	4.77	18
Control	34	0.13	262	1.62	1.1	30.0	3435	11.1	2.23	3.63	17
LSD (5 %)	1.5	0.018	44.1	0.255	0.16	2.95	62.9	0.76	0.772	0.815	1.4

LSD least significant difference

Table 4 Effect of the four selected bacterial isolates for their PGP and yield potential on chickpea under field conditions

Isolate	At 60 days after sowing						At crop harvest		
	Nodule number (plant^{-1})	Leaf area (cm^{-2} plant^{-1})	Leaf weight (g plant^{-1})	Pod number (plant^{-1})	Shoot weight (g plant^{-1})	Plant height (cm)	Stover yield (t ha^{-1})	Grain yield (t ha^{-1})	Total dry matter (t ha^{-1})
VBI-4	56	637	3.79	61	4.03	48.3	2.15	1.86	4.01
VBI-19	50	649	3.74	59	3.96	47.0	1.69	1.77	3.46
VBI-23	58	842	5.03	73	5.27	51.7	1.81	2.05	3.85
SBI-23	51	699	4.1	64	4.03	48.7	1.71	1.66	3.38
Control	49	626	3.71	59	3.96	47.0	1.68	1.67	3.34
LSD (5 %)	6.3	78.5	0.547	9.6	0.938	1.77	0.318	0.131	0.331

LSD least significant difference

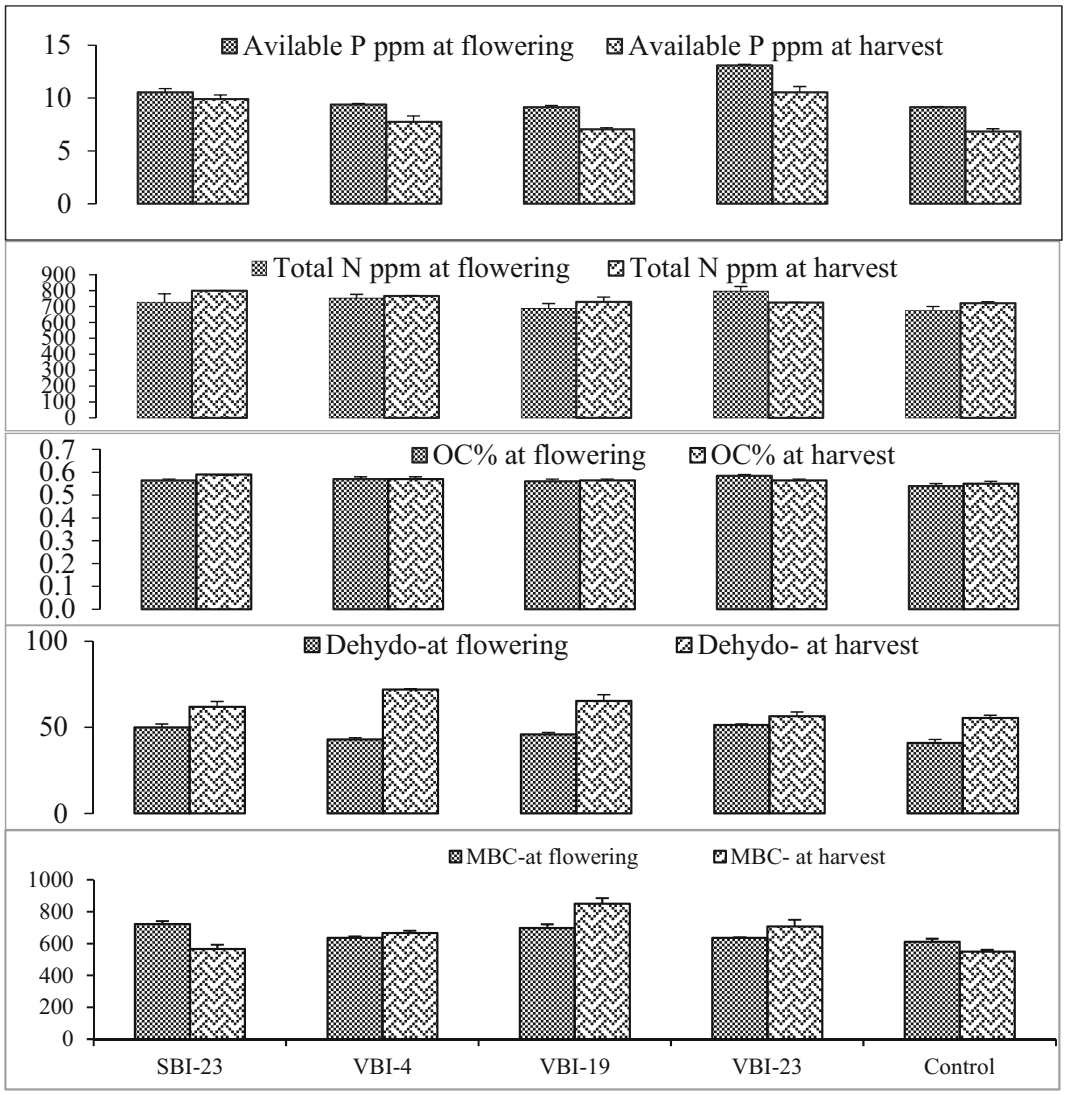

Fig. 3 Effect of the selected bacterial isolates on soil mineral and biological properties

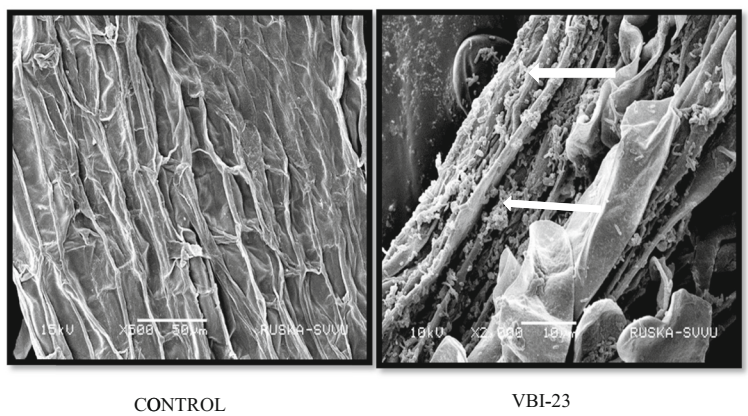

Fig. 4 SEM picture showing colonization of chickpea root by bacterial isolate VBI-23

played an important role in nutrient cycling for providing plant nutrition, reducing pathogen populations, increased soil organic matter, and total carbon, thus improving soil quality (Bulluck et al. 2002). Thus, the four selected bacterial isolates can be used for organic farming.

Colonization capability of selected bacterial isolates on chickpea roots

In the present study, all the four bacterial isolates were proved to colonize the roots of chickpea in SEM analysis without causing any damage to the chickpea roots (Fig. 4). Carbon fixed by plant photosynthesis is known to be partly translocated to the roots and released as root exudates (Bais et al. 2006). Compounds such as carbohydrates, amino acids, and organic acids are released in to the rhizosphere and thus attracting bacteria to colonize the roots (Walker et al. 2003). Hence, it can be concluded that the selected four bacterial isolates were attracted by the root exudates of chickpea and entered into the root system and colonized.

Conclusion

From this study, it was confirmed that all the four isolated *Bacillus* species possess good plant growth-promoting as well as biocontrol properties to increase the growth and yield enhancement in chickpea by both direct as well as indirect plant growth-promoting traits. Studies need be extended on multi-location trials in order to prove their plant growth-promoting ability on chickpea.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no financial/commercial conflicts of interest.

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