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Isolation, in vitro evaluation of *Bacillus* spp. against *Fusarium oxysporum* f.sp. *ciceris* and their growth promotion activity

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Abstract

Background: *Fusarium oxysporum* f.sp. *ciceris* is one of the potential threats to chickpea cultivation as it causes greater a yield loss under favorable conditions. Management of this disease is difficult because of its soil-borne nature and also existence of races. Fungicide application is not economical and not practical. Application of antagonistic bacteria like *Bacillus* species is one of the best approaches for its management.

Results: Ten *Bacillus* isolates were collected from healthy chickpea rhizosphere soil, identified and confirmed them as *Bacillus* based on the results obtained in Gram staining and biochemical characterization (up to species level). They were evaluated against *Fusarium oxysporum* f.sp. *ciceris* under in vitro conditions and selected two potential isolates (*Bacillus*-5 and *Bacillus*-7), screened for plant growth promotion properties and found that*Bacillus*-5 was able to solubilize phosphates, *Bacillus*-7 produced cellulases, both produced HCN and both were unable to produce IAA and chitinases. Identification of these two isolates was done by means of 16Sr DNA sequence analysis. Two universal primers such as 63F and 1387R were used which resulted in 1300 bp product. Blast analysis results indicated that they have more similarities with *Bacillus cereus*.

Conclusions: Two potential *Bacillus* isolates out of 10 were selected based on *in vitro* assay and subjected to study for plant growth promotion characters and found that*Bacillus*-5 solubilizes phosphates, *Bacillus*-7 produced cellulases and both produced HCN. Based on 16S rDNA analysis, these potential antagonists have more similar sequences of *Bacillus cereus*. Further, field efficacy studies need to do in future.

Keywords: Chickpea, Rhizosphere, Growth promotion, *Bacillus* spp., *Fusarium oxysporum* f.sp. *ciceris*, 6S r DNA

Background

Chickpea is the second most important pulse crop globally and greatly valued for its nutritional qualities and improvement of soil fertility status. *Fusarium* wilt of chickpea caused by *Fusarium oxysporum* f.sp. *ciceris* (Foc) is one of the major limitation to chickpea production worldwide. Under favorable conditions, *Fusarium* wilt epidemics can cause up to 100% loss in highly infested fields depending on varietal susceptibility and climatic conditions. The fungus is soil and seed borne; hence, the fungicide application is not effective and difficult to implement. Among the bioagents, *Bacillus* spp. are one of the best antagonists, as they show effective root colonization with multiple modes of action (Kloepper et al. 2004) and form endospores and proved as effective biocontrol agents in many crop plants. Identification of potential antagonists at species level is very important. In bacteria 16S rDNA sequence analysis is a very good tool of species identification. Hence, this experiment was planned to identify potential *Bacillus* isolates based on their in vitro efficacy, to study the ability of the isolates for plant growth promotion characters and identification at molecular level.

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Methods

This research work was conducted at Regional Agricultural Research Station (RARS), Nandyal, and molecular identification of potential antagonists was done at RARS, Tirupati, Andhra Pradesh.

Isolation of Fusarium oxysporum f.sp. ciceris

Pathogen (*Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matuo and Sato (Snyder and Hansen, 1940) was isolated from *Fusarium* wilt infected plants of chickpea, identified and confirmed in previous work (Venkataramanamma et al. 2022) which was utilized in the present research work.

Isolation of Bacillus from chickpea rhizosphere

Soil samples were collected during disease survey from healthy chickpea rhizosphere, composited and used for *Bacillus* (Domain: Bacteria, Phylum: Firmicutes, Class: Bacilli, Order: Bacillales and F: Bacillaceae) isolation. These soil samples (10 g) were heat-treated (80 °C) for 20 min to select resistant forms. Then the heat-treated soil was shifted to sterile distilled water of 90 ml and mixed completely by shaking the flask on a rotary shaker for 5 min, and 0.1 ml of each serial dilutions (10^{-3}–10^{-6}) was spread over nutrient agar plates (cooled) in triplicate and incubated at 30 ± 1 °C for 24–48 h. Rough colonies with waxy growth (1–4 mm dia.) and irregular spreading edge were attained and used for further bacterial identification. Microscopic observation (100x), Gram staining and biochemical characters (Hi *Bacillus* TM identification kit (KB013) (Himedia Laboratories Pvt. Ltd, Mumbai) were used for identification and confirmation. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls. The identification kit has 12 tests such as malonate, Voges-proskauers test, citrate, ONPG, nitrate reduction, catalase, arginin, sucrose, mannitol, glucose, arabinose, and trehalose, and manufacturer's instructions were followed for confirmation of the isolates as *Bacillus.*

Fungal-bacterial interactions in dual culture

After confirmation, two-day-old culture of *Bacillus* isolates grown on nutrient agar medium at 28 ± 2 °C was used for dual culture. A 5-mm disk of Foc (one-week-old culture) was kept at the center of PDA plate (Anjaiah et al. 1988); antagonistic bacteria were streaked individually on both sides of the pathogen at 2.5 cm distance leaving 2.0 cm from periphery in triplicate. CRD design was followed. Plates inoculated with pathogen served as control and these plates were incubated at 28 ± 2 °C. Mycelial percentage inhibition of test pathogen over control was calculated by using the formula given by Vincent (1927):

$$\text{Percent inhibition (I)} = \text{C} - \text{T} \times 100/\text{C}$$

where

I = Percentage inhibition of pathogen over control.
C = Radial growth (mm) of pathogen in control plate.
T = Radial growth (mm) of pathogen in treatment plate.

Plant growth promotion characters of potential antagonists

Potential *Bacillus* antagonists were screened for growth promotion activities like phosphate solubilization, production of IAA, HCN, cellulases and chitinases.

IAA production

Potential *Bacillus* isolates were screened for IAA production as per the procedure given by Kerkar et al. (2012). A loopful of potential *Bacillus isolates* were inoculated separately into pre-sterilized peptone broth containing 1% tryptophan in triplicate and incubated for 48 h. at 28 °C. One ml of Kovac's reagent (Qualigens, Mumbai) was added to all tubes including control after incubation and shacked after 15 min. Appearance of red ring at the top indicates positive of IAA production.

Phosphate solubilization

Both isolates of *Bacillus* were screened for phosphate solubilization on modified Pikovskaya's agar plate (Gupta and Sharma 1995). Bacteria were *inoculated* as one to three spots on the medium and production of clear halos indicated their ability to solubilize phosphates.

HCN production

For qualitative determination of production of hydrogen cyanide (HCN), Bakker and Schippers (1987) method was followed. The *Bacillus* isolates were inoculated on nutrient agar medium supplemented with glycine at the rate of 4.4 g/l. A Whitman filter paper no.1 soaked in 2% sodium carbonate and 0.5% picric acid was placed in the lid of each Petri dish. The plates were sealed with parafilm and incubated at 30 °C for 4 days. A change in color of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction, respectively, for the production of HCN.

Chitinase production

Chitinolytic ability of *Bacillus* isolates was assessed by keeping a spot of 48-h-old culture in the center on water agar incorporated with 0.2% colloidal chitin (Berger and Reynolds 1958) and incubated at room temperature for 4 days and development of a hydrolytic zone (clearing zone) is a sign for chitinase production.

Cellulases

Cellulolytic ability of bacteria was assessed by using Mandels and Reese medium (Mandels and Reese 1957) containing CMC salt. All the inoculated plates were stained with 1% Congo red solution for 15 min after incubation at 28 °C for 48 h, and distained with 1 M NaCl for 15 min (Teather and Wood 1982). The degradation zones around the bacteria indicated positive for cellulose production.

Molecular characterization of potential Bacillus isolates by 16S rDNA

Extraction of DNA

Bacterial genomic DNA isolation kit from M/s Medox, Chennai was used for extraction of DNA from potential *Bacillus* isolates. The quality and quantity of DNA were verified on 1% Agarose gel and by Nano drop spectrophotometer. The 16S rDNA sequence was employed for identification of potential *Bacillus* isolates. The primers 63F (CAGGCCTAACACATGCAAGTC) and 1387R (GGG CGGATGTGTACAAGGC) were used for PCR amplification (Marchesi et al., 1998). Amplified product size of 1300 bp approximately was produced by both primers in two isolates under the study as expected. PCR technique has been standardized as a part of this and the following conditions were used for the amplification of 16S rDNA.

Stage—I	Initial denaturation	94 °C for 4 min
Stage—II	Denaturation	94 °C for 1.0 min
	Annealing	55.4 °C for 1 min
	Extension	72 °C for 1.5 min
	Number of cycles: 35	
Stage—III	Final extension	72 °C for 5 min

The amplified products were visualized in 1% agarose gel and PCR products were sent for sequencing to M/s Eurofins Genomics India private limited, Bengaluru. Both forward and reverse sequences were aligned and a consensus sequence was obtained. Then, BLAST program was used to ascertain the species identity of *Bacillus* isolates in NCBI GenBank.

Results

Isolation and identification of Bacillus isolates

A total of 10 *Bacillus* isolates were collected from different parts of Andhra Pradesh. The colonies which exhibited rough, waxy growth and irregular spreading margin on nutrient agar media were selected and purified by re-streaking on same medium. Ten isolates (Fig. 1) out of 12 showed positive reaction upon Gram staining (Fig. 2). The results obtained in biochemical test (Hi *Bacillus* TM identification kit (KB013)) confirmed them as genus *Bacillus* (Fig. 3) and based on the results obtained, *Bacillus*-2 and 9 were identified as *B. thuringiensis*, *Bacillus*-6 and *Bacillus*-8 as *B. megaterium*, *Bacillus*-5 and *Bacillus*-7 as *B. cereus*, *Bacillus*-1, *Bacillus*-10 and *Bacillus*-4 as *B. pumilis* and *Bacillus*-3 as *B. subtilis*, but molecular confirmation was done only for potential antagonists.

Bacillus against Fusarium oxysporum f.sp.ciceris

The in vitro evaluation test results revealed significant differences in inhibition growth of pathogen with different isolates of *Bacillus* and varied from 2.3 to 74.36%. *Bacillus*-7 isolate exhibited highest inhibition of pathogen growth to an extent of 74.36% (Table 1 and Fig. 4), and it was followed by *Bacillus*-5, *Bacillus*-6, *Bacillus*-4 which showed inhibition percentage of 71.63, 57.73 and 52.89% respectively, over control. *Bacillus*-7 and *Bacillus*-5 were non-significant with each other in inhibition percentage. *Bacillus*-3 recorded inhibition percentage of 51.85 and it was non-significant with *Bacillus*-4. The lowest percentage of inhibition was observed in the isolate *Bacillus*-2 (2.3), followed by *Bacillus*-9 (6.2) which were non-significant with each other. *Bacillus*-8 also reduced pathogen growth to the extent of 47.66%.

Overgrowth of pathogen on *Bacillus* was observed in *Bacillus*-1, *Bacillus*-2, *Bacillus*-9 and *Bacillus*-10. Among the tested *Bacillus* isolates *Bacillus*-5 exhibited highest inhibition zone of 14 mm, followed by *Bacillus*-3 and *Bacillus*-6 formed an inhibition zone of 8 mm and the least inhibition zone of 5 mm was formed by *Bacillus*-4 and *Bacillus*-8. Pigmentation was produced by Foc in dual cultures of *Bacillus*-3, *Bacillus*-6, *Bacillus*-7, *Bacillus*-8 and changed in the mycelium color was also observed in some dual cultures. It might be due to the fungistatic metabolites secreted by *Bacillus* isolates.

Plant growth promoting characters of bacterial antagonists

The potential *Bacillus* isolates studied for plant growth promotion properties are presented in Table 2.

The results indicated that only *Bacillus*-5 was able to solubilize phosphates, *Bacillus*-7 produced cellulases and both produced HCN. But none of them produced IAA and chitinases.

Molecular identification of Bacillus isolates

Amplification of the 16S rDNA region from isolates of *Bacillus*-5 and *Bacillus*-7 with primers 63F and 1367R yielded products of approximately 1300 bp (Fig. 5) and sequenced the PCR products of both isolates. The sequences of two *Bacillus* isolates were compared (BLAST analysis) with other sequences available in the GenBank data (Table 3) base to identify the organism.

Results obtained in sequence analysis revealed that both potential *Bacillus* isolates had more sequence

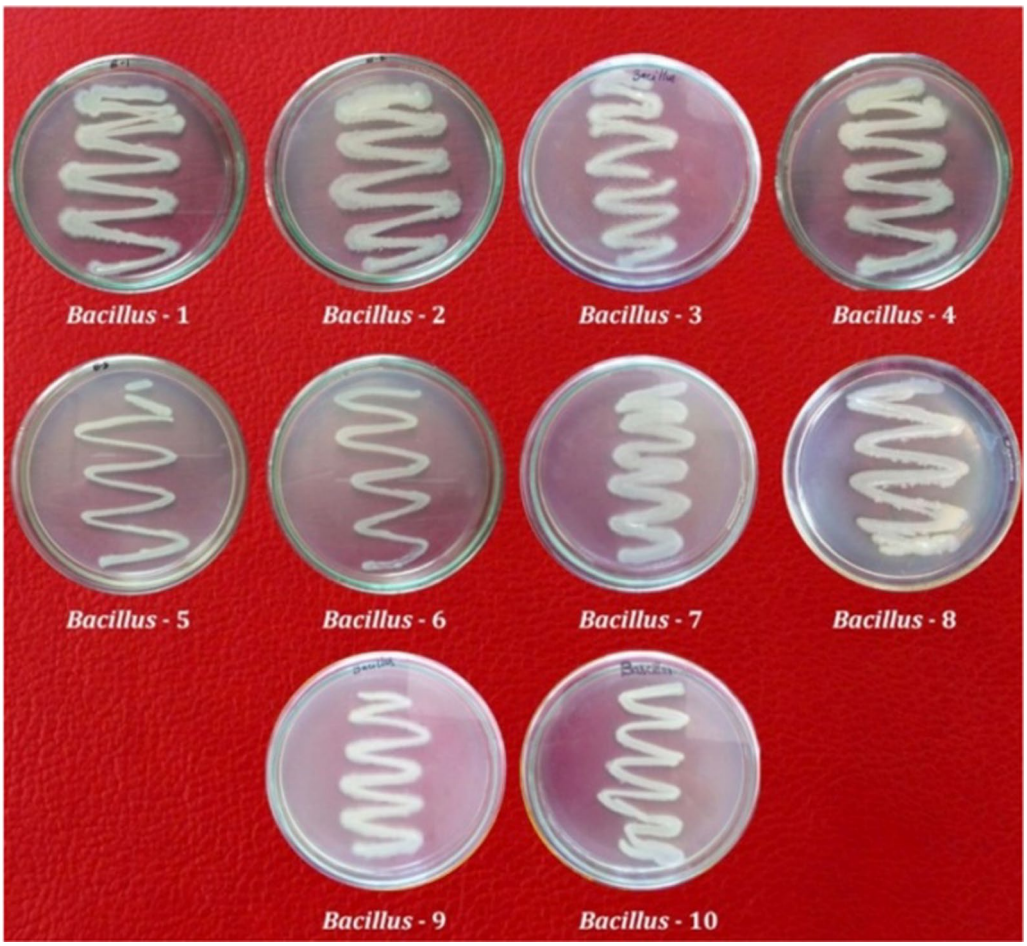

Fig. 1 Pure cultures of *Bacillus* isolates

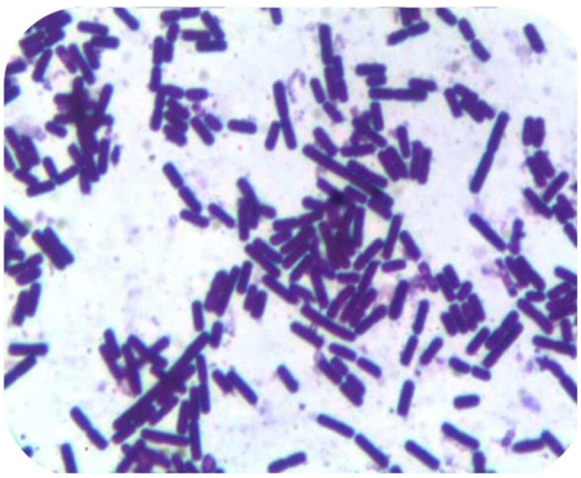

Fig. 2 Gram positive reaction of *Bacillus* isolates

similarities as *Bacillus cereus* Frankland&Frankland 1887 strains.

Discussion

Isolation and identification of native *Bacillus* spp. is an important step to have effective biocontrol against soil-borne pathogens like *Fusarium*. In the present experiment, 10 *Bacillus* species were isolated. Similarly, Smitha et al. (2015) isolated 30 *Bacillus* spp. from chickpea rhizosphere and screened against Foc and *Rhizoctonia bataticola* (Taub.) Butler. (Synonym: *Macrophomina phaseolina* (Maubl.) Ashby.) under in vitro conditions. Pankajkumar et al. (2012) used Hi25TM Enterobacteriaceae Identification Kit and HiCarbohydrateTM Kit (Himedia Laboratories Pvt. Ltd, Mumbai)for physiological and biochemical characterization of selected *Bacillus* isolates.

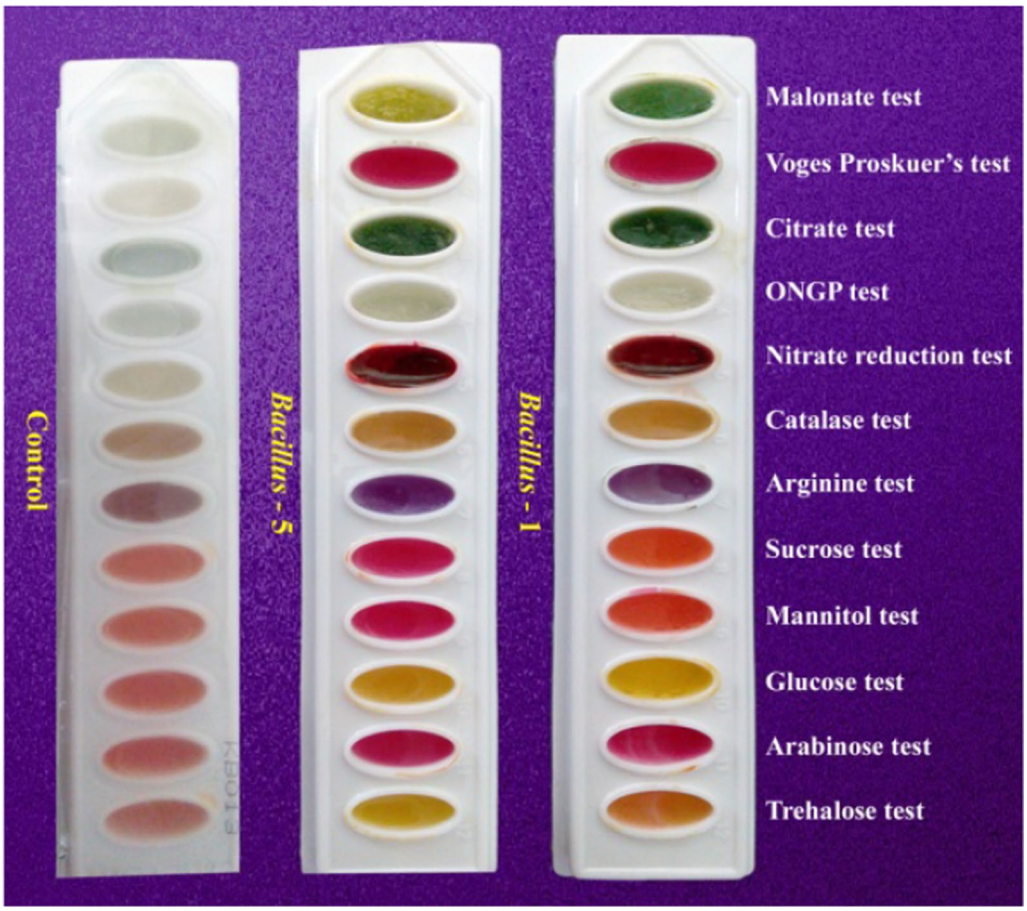

Fig. 3 Biochemical tests for identification of *Bacillus* isolates

Table 1 In vitro antagonistic potential of *Bacillus* isolates against *Fusarium oxysporum* f.sp. *ciceris*

S. no	Isolate name	Radial growth (mm)	Inhibition (%) over control
1	*Bacillus*-1	52.00	18.07 (25.16)
2	*Bacillus*-2	62.00	02.30 (8.73)
3	*Bacillus*-3	30.55	51.85 (46.08)
4	*Bacillus*-4	29.90	52.89 (46.68)
5	*Bacillus*-5	18.00	71.63 (57.85)
6	*Bacillus*-6	26.82	57.73 (49.68)
7	*Bacillus*-7	16.27	74.36 (57.85)
8	*Bacillus*-8	33.21	47.66 (43.68)
9	*Bacillus*-9	54.23	14.54 (22.43)
10	*Bacillus*-10	59.51	06.20 (14.37)
	Control		0.00
	Sem ±		6.95
	CD (5%)		2.56
	CV (%)		4.31

Different inhibition percentage was observed between *Bacillus* isolates and Foc under in vitro conditions. Our results are in accordance with Zaim et al. (2013) who tested 29 potential rhizobacteria isolates against Foc, found five *Bacillus* isolates were potential and recorded inhibition percentage in the range of 25.63 to 71.11 and also observed inhibition zone in *Bacillus* and Foc interactions. Similarly, Anusha et al. (2019) isolated a total of 40 bacterial isolates from chickpea rhizosphere, tested against Foc and out of which 5 isolates of each were identified as *Streptomyces* spp and *Bacillus* spp based on morphological, biochemical and 16S rDNA analysis.

Indole acetic acid (IAA) plays a central role in plant growth and development. But in the present study none of them produced IAA, one isolate (*Bacillus*-5) solubilizes phosphates. The two bacterial isolates produced HCN. Rouag et al. (2019) conducted a study on plant growth promoting characters of six potential *Bacillus* isolates against Foc and found that all produced IAA, four isolates of each produced chitinases, cellulases, and

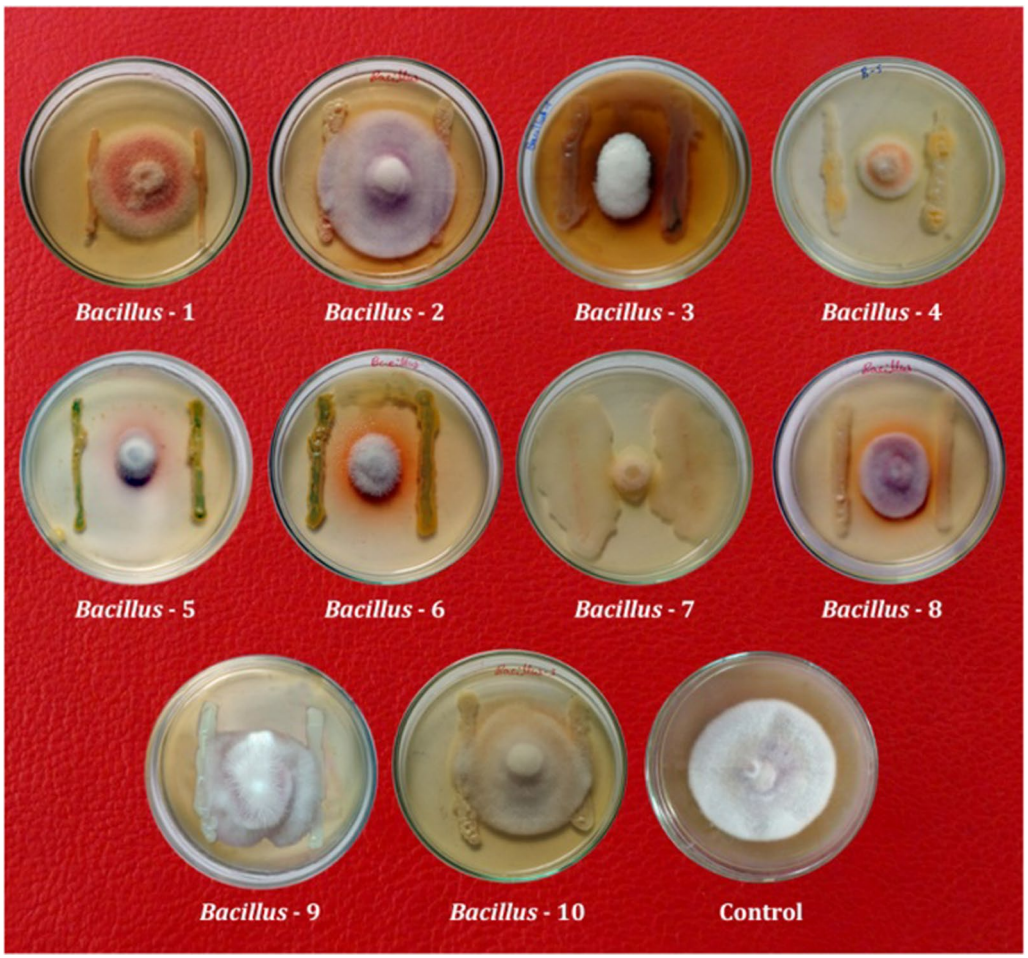

Fig. 4 *In vitro* evaluation of *Bacillus* isolates against *Fusarium oxysporum* f.sp. *ciceris*

Table 2 Screening of potential *Bacillus* antagonists for plant growth promotion characters

Isolates' name	IAA production	Phosphate solubilization	HCN	Chitinases	Cellulases
Bacillus-5	–	+	+	–	–
Bacillus-7	–	–	+	–	+

one isolate solubilizes phosphates. Phosphate solubilization property of bacteria helps to promote plant growth. HCN produced by bacteria isolated from chickpea rhizosphere promotes plant growth directly, indirectly and synergistically (Joseph et al. 2007). Kremer and Souissi (2001) reported that approximately 32% of bacteria from a collection of over 2000 isolates were cyanogenic, produced HCN from trace concentration to > 30 n moles/mg cellular protein. Usually, the host plant is not affected by HCN generated by bacteria. *Bacillus* can operate as a bioagent, whereas cyanide acts as a metabolic inhibitor to discourage competition predation or acts as a safeguard from phytopathogenic fungi. Ghodsalavi et al. (2013) reported that approximately 90% of *Bacillus* spp. are able to produce HCN. Similarly, in the present experiment, HCN produced by two isolates might be played an important role in pathogen suppression by *Bacillus*.

In the present experiment chitinase enzyme activity was not detected and cellulose activity was found only in one bacterial isolate, *i.e.*, *Bacillus*-7 (Table 2). Cellulase play a major role in disease suppression but also promotes plant growth and organic matter decomposition. Similarly, Ahmad et al. (2008) found that 72 bacterial isolates belonging to genera of *Azotobacter*, fluorescent Pseudomonas, *Mesorhizobium* and *Bacillus* when tested for plant growth promoting characters, all showed the production of IAA, ammonia, solubilization of phosphates and none of them was able to hydrolyzed chitin. Vijaya et al. (2011) evaluated *Bacillus* strain MBI 600 for plant growth promotion characters and observed that

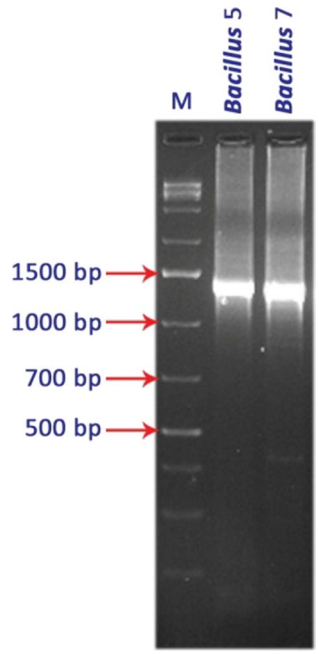

Fig. 5 PCR amplification of 16S rDNA from two potential isolates

it was unable to produce chitinases, cellulases and produced only siderophores. Datta et al.(2011) suggested that growth promotion usually takes place by synthesis of some phytohormones, production of siderophores and disease control by antibiotics, hydrogen cyanide (HCN) is an added advantage of these bacteria. In the present experiment, the two potential bacterial isolates produced HCN, one isolate solubilize phosphates and one produced cellulases and both were not produced IAA, chitinases.

Molecular analysis of ITS region of 16S r DNA was used for identification of potential *Bacillus* species, from the results, it was known that two isolates have close similarities with *B.cereus* strains. Kumbar et al. (2017) suggested that 16S rDNA sequencing was used for identification of 13 *Bacillus* isolates from Karnataka as *Bacillus subtilis* (Ehrenberg 1835, Cohn 1872). Wahyudi et al. (2010) studied the inhibition activity of 22 species of *Bacillus* on *F.oxysporum* (Schlecht as emended by Snyder and Hansen), *Rhizoctonia solani* (Kuhn 1858), *Sclerotium rolfsii* Sacc. and six efficient strains were selected and identified three strains as *B.cereus*, two as *B. subtilis* and one as *Lysinibacillus fusiformis* (Priest et al. 1988) based on 16S r DNA analysis and obtained 1300 bp product by using the primers 63F and 1867R. The efficacy of *Bacillus* on *Fusarium* was reported by Thangavelu and Gopi (2015) who evaluated different combinations of rhizospheric and endophytic bacteria including *Bacillus cereus* and *Pseudomonas putida* (Trevisan 1889, Migula 1895) on *Fusarium* wilt of Banana and found all the treatments were effective. Raminez et al. (2021) reported the efficacy of *Bacillus cereus* MH 778,713 isolate on suppression of *F. oxysporum* growth under in vitro and in vivo (green house) conditions.

Conclusions

Two *Bacillus* isolates out of 10 were selected against Foc and studied plant growth promotion characters and identified that both isolates produced HCN, one produced cellulases and one solubilizes phosphates. Both the isolates were identified as *Bacillus cereus* based on 16S rDNA gene sequence analysis. Further, the potentiality of these two isolates should be evaluated under field conditions in future.

Abbreviation

Foc: *Fusarium oxysporum* F.sp. *ciceris*.

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Author contributions

KVR performed the experiments. BVBR guided to perform the experiment accurately. RSJ, guided in calculation of B:C ratio in this research article. VJL provided seed material to carry out this research. LRJ helped in writing this research article. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Table 3 BLAST analysis of 16S r DNA region of *Bacillus* isolates with other sequences in data base

S. no	Isolates under study	Species name from data base	Max score	Total score	Query cover (%)	Identity (%)	Accession number
1	*Bacillus*-5	*Bacillus cereus* strain	1393	1592	84	93	KC626001
2	*Bacillus*-5	*Bacillus cereus* strain	1393	1592	84	93	KC248214
3	*Bacillus*-7	*Bacillus cereus* strain	739	862	64	84	KJ874356
4	*Bacillus*-7	*Bacillus cereus* strain	737	934	67	84	GQ280806

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors have no competing interest in publication of this research article.

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