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# Exploiting novel rhizosphere *Bacillus* species to suppress the root rot and wilt pathogens of chickpea

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**Thirty isolates of *Bacillus* were collected from chickpea rhizosphere and screened for their *in vitro* inhibition against root rot (*Rhizoctonia bataticola*) and wilt (*Fusarium oxysporum* f.sp. *ciceri)* pathogens and growth promotion of chickpea. Based on the *in vitro* inhibition and growth promotion tests, the best eight isolates were selected and PCR-based detection of antibiotics genes *viz.*, surfactin, iturin, fengycin and bacillomycin D was carried out. The isolate which produced all these antibiotics and showed maximum *in vitro* inhibition (CaB 5) was further used for crude antibiotics extraction and inhibition assays. The presence of antibiotics in crude extract was detected through TLC. The inhibitory effect of the crude extract was proved through agar-well diffusion assay and spore germination inhibition test. From this study, it was inferred that the *Bacillus subtilis* strain CaB5 was promising in inhibiting the root rot and wilt pathogens of chickpea and enhance seedling vigour.**

**Key words:** Biological control, plant growth promotion, surfactin, iturin, fengycin.

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume with 96% of the total cultivated area in the developing countries. It is the premier pulse crop of Indian subcontinent. India is the largest producer as well as consumer of chickpea in the world. About 65% of the global chickpea area falls in India, corresponding to 68% of the global production (FAOSTAT, 2012). In India, chickpea is grown in an area of 8.3 Mha with a production of 7.7 MT and productivity of 928 kg/ha (Directorate of Economics and Statistics, 2011-12). Despite its economic importance, the productivity is low owing to many biotic and abiotic stresses. Chickpea is affected by many soil-borne diseases of which the root rot pathogen *Rhizoctonia bataticola* (Taub). Butler and vascular wilt fungus *Fusarium oxysporum* f.sp. *ciceri* are the most important in Indian subcontinent. At field level, these diseases can be managed to some extent by using seed dressing fungicides and chemical sprays. Of late, there has been a shift from chemical to biological control methods owing to the toxicity hazards and environmental pollution due to overuse of chemicals.

*Bacillus* species are outstanding biocontrol agents as they show effective root colonization, multiple modes of action and promising ability to sporulate (Kloepper et al.,

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2004). Cyclic lipopeptides of the surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* species. *Bacillus subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and potential to produce more than two dozen of structurally diverse antimicrobial compounds (Stein, 2005). Turner and Backman (1991) found that *Bacillus* sp. colonized the root surface, increased plant growth and caused lysis of fungal mycelia. Their endospore-forming ability also makes these bacteria one of the best candidates for developing efficient biopesticide products from a techno-logical point of view (Gordillo and Maldonado, 2012). Hence an effort was made to assess the *in vitro* efficacy of *Bacillus* spp. against root rot and wilt pathogens of chickpea.

## MATERIALS AND METHODS

### Isolation of the pathogens

Chickpea plants showing typical symptoms of root rot and wilt were collected from the fields and used for isolation of the pathogens. Isolation was made from collar and stem regions in the case of *Fusarium oxysporum f.sp. ciceri* and root and collar regions were used for isolating *Rhizoctonia bataticola*. The tissues were washed in running tap water, cut into small bits of 5-10 mm, surface sterilized with 0.1% mercuric chloride for 30 s, blotted dried on sterile filter paper and plated on potato dextrose agar (PDA) medium. The plates were incubated at 22-25°C for 2-3 days and actively growing mycelia transferred to PDA slants by hyphal tip method. The pathogenicity of both *F. o. f.sp. ciceri* and *R. bataticola* were proved as per Koch's postulates.

### Collection of *Bacillus* strains

A survey was conducted in the major chickpea growing areas of Tamil Nadu *viz*., Coimbatore, Tirupur and Dindigul during the period from October to December 2013. Rhizosphere soil samples were collected and *Bacillus* spp. were isolated by serial dilution plate technique. Ten grams of soil was added to 90 ml of sterile distilled water and subjected to 80°C for 20 min in water bath so that only the spores of bacteria remained in the suspension. This was serially diluted up to $10^{-7}$ and plated on nutrient agar (NA) plates. These isolates were purified and maintained on NA slants.

### *In vitro* evaluation of *Bacillus* strains

Thirty rhizosphere *Bacillus* strains were tested for their *in vitro* efficacy against *F. o. f.sp. ciceri* and *R. bataticola* by dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked at one side of 90 mm Petri dish (1 cm from the edge of a plate) with PDA medium and mycelial disc (5 mm diameter) of actively growing (seven days old) culture of the pathogens placed on the opposite side in the Petri dish perpendicular to the bacterial streak. The experiment was laid out in completely randomised design with three replications for each treatment. The plates were incubated at room temperature (28 ± 2°C) for seven days and the mycelial inhibition of pathogen over control was calculated according to the formula given by Vincent (1947).

PI = (C-T) / C x 100

Where, PI– Inhibition percentage, C– Rate of growth of pathogen in control and T– Rate of growth of pathogen in treatment

### Plant growth promotion tests

The eight bacterial isolates which showed *in vitro* inhibition of the pathogens were inoculated in a conical flask with 100 ml of nutrient broth. Required quantity of chickpea seeds (cv. CO4) were soaked in bacterial suspension containing 3 x $10^8$ cfu $ml^{-1}$ for 2 h and shade dried. The seeds soaked in sterile water served as control. Plant growth-promoting activity of rhizospheric bacterial isolates was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Ten seeds were kept over the pre-soaked germination paper. The seeds were held in position by placing another pre-soaked germination paper and gently pressed. The polythene sheet along with seeds was then rolled and incubated in growth chamber for 10 days. Three replications were maintained for each treatment. The root and shoot length of individual seedlings were measured and the germination percentage of seeds were calculated.

Plant growth promotion was also tested by pot culture method. Bacterized seeds were sown in pots. Fifteen seeds were maintained for each treatment. The root and shoot length of individual seedlings were measured and germination percentage of seeds was calculated. The vigour index was calculated by using the formula as described by Baki and Anderson (1973).

Vigour index = per cent germination x seedling length (shoot length + root length)

### Detection of antibiotic genes of *Bacillus* strains

To confirm that the selected strains have the capacity to produce antibiotics, the genomic DNA was isolated for antibiotic gene detection through PCR. Genomic DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method described by Knapp and Chandlee (1996), with slight modifications (Melody, 1997). The antibiotic genes surfactin, iturin, fengycin and bacillomycin D were amplified using the primers described below.

#### *Surfactin*

The forward primer SUR3F (5'ACAGTATGGAGGCATGGTC 3') and reverse primer SUR3R (5' TTCCGCCACTTTTTCAGTTT 3') were used for amplification of surfactin gene (440 bp) (Ramarathnam, 2007). PCR amplification was performed in a thermocycler using the following conditions: Initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1min (primer extension) and final extension at 72°C for 10 min.

#### *Iturin A*

The forward primer ITUD1F (5' GATGCGATCTCCTTGGATGT 3') and reverse primer ITUD1R (5' ATCGTCATGTGCTGCTTGAG 3') were used for amplification of iturin A gene (648 bp) (Ramarathnam, 2007). The PCR reaction conditions were initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

#### *Fengycin D*

The forward primer FEND1F (5'TTTGGCAGCAGGAGAAGTTT3') and

reverse primer FEND1 R (5'GCTGTCCGTTCTGCTTTTTC3') were used for amplification of fengycin gene (964 bp) (Athukorala et al., 2009). PCR amplification conditions used were as follows: Initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension of 72°C for 10 min.

***Bacillomycin D***

The forward primer BACC1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer BACC1R (5'CGCTGATGACTGTTCATGCT3') (Operon, Inc., Alameda, CA) were used for amplification of bacillomycin D gene (876 bp) (Ramarathnam, 2007).

PCR amplification conditions were initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 62°C for 1 min (annealing), 72°C for 1 min (primer extension) and a final extension of 72°C for 10 min.

**Agarose gel electrophoresis and gel documentation**

Agarose gel electrophoresis was performed based on the method given by Sambrook et al. (1999) to check the quality of DNA and also to separate the products amplified through the polymerase chain reaction. After separation on 1.5% agarose gel at 50 UV, the PCR products were stained with ethidium bromide (0.5 µg/ml), photographed and analyzed using a gel documentation system.

**Extraction of crude antibiotics**

Based on the presence of antibiotic genes, *in vitro* inhibition and growth promotion, the best isolate was selected for extraction of crude antibiotics. The crude antibiotics were extracted as per the method described by McKeen et al. (1985). The bacteria was grown in Landy's medium (20 g D-glucose, 5 g L-glutamic acid, 1.02 g $MgSO_4 \cdot 7H_2O$, 1 g $KH_2PO_4$, 0.5 g KCl and 1 ml of trace element solution (0.5 g $MnSO_4 \cdot 4\ H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 0.015 g $FeSO_4 \cdot 7H_2O$ in 100 ml of water per litre). The pH of the media was adjusted to 6.0 to 6.2 with 5 N NaOH. A loopfull of 24-h old bacterial culture was inoculated into 100 ml of Landy medium and the inoculated flasks were incubated on a shaker at 170 rpm and 30°C for 3 days. The production medium was centrifuged for 20 min at 12,000 rpm to remove bacterial cells. The antibiotics were precipitated from the supernatant by adjusting the pH to 2.5 with concentrated HCl. This was centrifuged for 10 min at 12,000 rpm. The pellet containing the active fraction was extracted thrice with 80% ethanol. The ethanol extract was dried under vacuum at 55°C on a rotary evaporator. Inactive substances were removed by sequential extraction with ethyl acetate and acetone. The resulting residue was dissolved in 80% ethanol and stored at 4°C.

**Thin layer chromatography (TLC) of crude antibiotics**

The 80% ethanol fraction with antibiotic activity was spotted onto 20 × 20-cm silica gel plates. The plates were developed with ethanol : water (2:1, v/v) and the bands were visualized with UV light and also by spraying 0.2 g of ninhydrin per 100 ml of 95% ethanol and heated at 110°C for 5-10 min to detect ninhydrin positive materials.

***In vitro* assay using crude antibiotics**

The efficacy of the crude antibiotics in pathogen inhibition was tested by agar well diffusion method and by cavity slide technique.

**Mycelial growth inhibition assay**

The agar well diffusion assay, as reported by Tagg and McGiven (1971) and modified by Islam et al. (2012) was used to determine the antagonistic activity of crude antibiotic extract. PDA medium was poured into each sterile Petri dish, followed by placement of 5 mm diameter mycelial disc of the pathogen at the centre of the plates. A 7 mm diameter well was made by punching the agar with a sterile cork borer on the corner of the plate in four places with equal distance. Then the crude antibiotic extract from the selected isolate CaB5 was poured into the wells at the rate of 50 µl per well and incubated for 96 h at 28±2°C. The inhibitory activity was expressed as the percent growth inhibition, as compared to the control with solvent alone, according to the following formula:

Growth inhibition (%) = (DC − DT)/DC × 100.

where, DC, diameter of fungal colony in control; and DT, diameter of fungal colony with treatment (Pandey et al., 1982).

**Spore germination inhibition assay**

Effect of crude antibiotics on conidial germination was tested by cavity slide technique. One drop of the culture filtrate of the isolate *B. subtilis* CaB5 was placed in a sterile cavity slide and allowed to air dry. One drop of the conidial suspension of the pathogen ($5\times10^4$ conidia/ml) was added and mixed thoroughly. Conidial suspension + sterile distilled water served as control. The slides were kept in moist growth chamber and incubated at 25±2°C. Observation on conidial germination of pathogen was recorded at 6, 24, and 36 h after incubation by microscopic examination.

**Statistical analysis**

The data were statistically analyzed (Rangasamy, 1995) using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). The percentage values of the *in vitro* inhibition were arcsine transformed. Data were subjected to the analysis of variance (ANOVA) at two significant levels (< 0.05 and < 0.01) and means were compared by Duncan's multiple range test (DMRT).

## RESULTS AND DISCUSSION

Biological control using antagonists provides an alternative to chemicals in plant disease management. The myco-parasitic potential of *Bacillus* spp. is well documented (Johri et al., 2003; Saharan and Nehra, 2011). In the present study, the initial screening of 30 isolates of *Bacillus* collected from different chickpea rhizosphere soils resulted in the selection of eight isolates which showed inhibition of both root rot (*R. bataticola)* and wilt (*F. oxysporum* f.sp. *ciceri*) pathogens of chickpea under laboratory conditions. The *Bacillus* genus specific primers BCF1 (CGGGAGGCAGCAGTAGGGAAT) and BCR2 (CTCCCCAGGCGGAGTGCTTAAT) amplified a fragment of approximately 546 bp corresponding to the region of the 16S-23S rRNA intervening sequence for *Bacillus* sp. Among the eight isolates, the *Bacillus* strain CaB5 (*Cicer arietinum Bacillus* 5) showed 53.3% inhibition of *F. oxysporum* f.sp. *ciceri* and 58.5% inhibition of

**Table 1.** *In vitro* antagonistic activity of *Bacillus* isolates against *F. oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola*.

| Bacillus isolate | Percent inhibition over control of *F. oxysporum f.*sp. *ciceri* | Percent inhibition over control of *R. bataticola* |
|---|---|---|
| CaB1 | 37.040 (37.4849)$^{d}$ | 40.370 (39.4444)$^{cd}$ |
| CaB2 | 32.590 (34.8070)$^{e}$ | 42.960 (40.9503)$^{c}$ |
| CaB3 | 43.703 (41.3802)$^{bc}$ | 37.780 (37.9230)$^{d}$ |
| CaB4 | 37.040 (37.4849)$^{d}$ | 31.113 (33.8989)$^{e}$ |
| CaB5 | 53.333 (46.9130)$^{a}$ | 58.517 (49.9093)$^{a}$ |
| CaB6 | 44.813 (42.0208)$^{b}$ | 52.220 (46.2734)$^{b}$ |
| CaB7 | 40.370 (39.4444)$^{cd}$ | 30.370 (33.4373)$^{e}$ |
| CaB8 | 30.370 (33.4373)$^{e}$ | 24.443 (29.6256)$^{f}$ |
| Control | - (0.5730)$^{g}$ | - (0.5730)$^{g}$ |

Values are mean of three replications. Data in parenthesis are arcsine transformed values. Data followed by the same letter in a column are not significantly different according to Duncan's multiple range test at p = 0.05.

**Table 2.** Effect of *Bacillus* isolates on chickpea seedling growth.

| Isolate | Vigour index | |
|---|---|---|
| | Roll towel | Pot culture |
| CaB1 | 2890.00$^{b}$ | 1908.93$^{bc}$ |
| CaB2 | 2895.00$^{b}$ | 2046.14$^{b}$ |
| CaB3 | 1696.00$^{d}$ | 2000.00b |
| CaB4 | 2340.00$^{c}$ | 1786.16$^{cd}$ |
| CaB5 | 3230.00$^{a}$ | 2412.84$^{a}$ |
| CaB6 | 2329.20$^{c}$ | 2267.71$^{a}$ |
| CaB7 | 2550.00$^{c}$ | 2044.72$^{b}$ |
| CaB8 | 2349.00$^{c}$ | 2052.60$^{b}$ |
| Control | 1641.50$^{d}$ | 1622.22$^{d}$ |

Values are mean of three replications; Control: Seeds treated with water instead of bacteria; Data followed by the same letter in a column are not significantly different according to Duncan's multiple range test at p = 0.05.

*R. bataticola* (Table 1) which was significantly higher as compared to other isolates. The results are in concordance with the findings of Zaim et al. (2013) who has reported *in vitro* mycelial inhibition of *F. oxysporum* f.sp. *ciceri* by antagonistic *Bacillus* spp.

Apart from mycelial growth inhibition, plant growth promotion is an important trait of soil microorganisms for improving crop productivity. The eight selected isolates were tested for growth promotion of chickpea by standard roll towel method and by pot culture studies. The results of the study revealed that all the eight isolates increased the vigour index of chickpea seedlings as compared to the control (Table 2). The plant growth promoting activity of *Bacillus* has been previously reported by Rajendran et al. (2007).

## Detection of antibiotic genes of *Bacillus* isolates

Production of antimicrobial compounds serves as a determinant to decide the ability of an organism to control plant diseases. The beneficial rhizobacteria *B. subtilis* is one of the best biocontrol agents because it produces lipopeptides *viz;* fengycin, iturin and surfactin which displayed multifaceted biocontrol activity against plant pathogens (Ongena and Jacques, 2008). These anti-microbial cyclic lipopeptides (LPs) *viz*; surfactin, iturin and fengycin are specifically interesting because of their high surface activities and antagonistic potential (Kim et al., 2004).

Specific PCR primers were employed for the detection of biosynthetic genes of multimodular enzymes, the peptide synthetases, involved in the synthesis of antifungal lipopeptides. The PCR amplified products, after separation in agarose gel electrophoresis followed by gel documentation, revealed the presence of surfactin, iturin, fengycin and bacillomycin D genes in the *Bacillus* strains. The surfactin gene was amplified at 440 bp, iturin gene at 648 bp, fengycin at 986 bp and bacillomycin D gene at 875 bp. Generally, many members of the *Bacillus* spp. are known producers of lipopeptides belonging to the surfactin, iturin and fengycin families.

The genes identified in each isolate in the present study are furnished in Table 3. Four of the eight isolates were found to produce all the four antibiotic genes. The isolate CaB5 which had all the four antibiotic genes and which showed maximum *in vitro* inhibition was selected for further studies. The 16s rRNA of the isolate CaB5 was sequenced and identified as *Bacillus subtilis* and the gene sequence is submitted at NCBI with the Accession No: KP412481.

## Extraction of crude antibiotics and TLC

The production of lipopeptides by the *Bacillus* strain CaB5 was confirmed by extraction of crude antibiotics and tested by TLC. The results of TLC indicated the presence of two bands by UV visualization with one at R*f* value 0.4 and another at R*f* value 0.7. On spraying with 0.2% ninhydrin, bands were visible at 0.08, 0.67, 0.72

**Table 3.** List of antibiotic genes identified in each *Bacillus* isolate.

| Isolates | Surfactin | Iturin A | Fengycin D | Bacillomycin D |
|---|---|---|---|---|
| CaB1 | + | - | - | + |
| CaB2 | + | + | + | + |
| CaB3 | + | + | + | + |
| CaB4 | + | + | - | + |
| CaB5 | + | + | + | + |
| CaB6 | + | + | + | + |
| CaB7 | - | + | + | + |
| CaB8 | - | + | + | - |

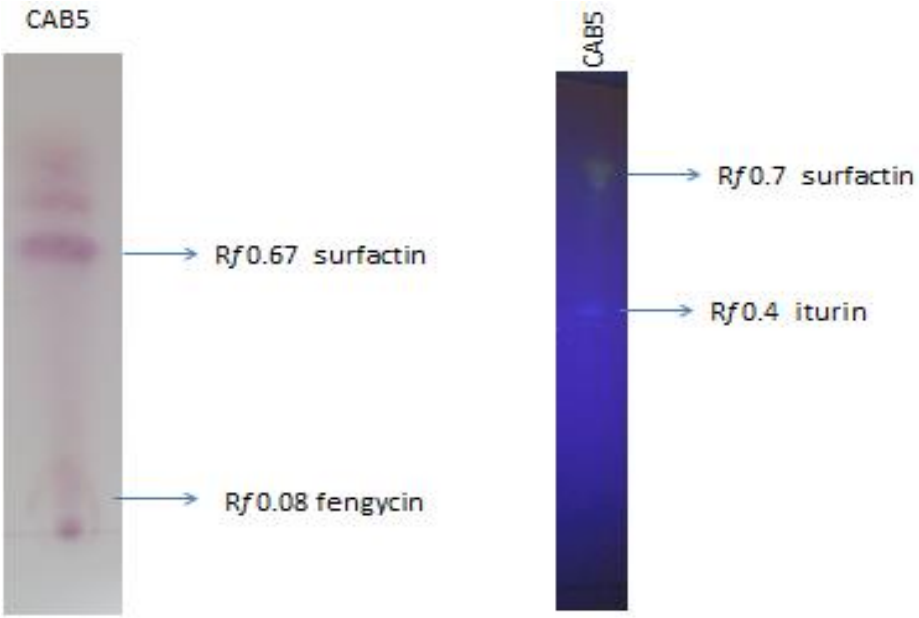


**Figure 1.** Thin layer chromatography of crude antibiotics of the *Bacillus* strain CaB5.

and 0.75 R*f* values (Figure 1). The crude antibiotics of *Bacillus amyloliquefaciens* PPCB004 developed spots with R*f* between 0.08 and 0.2 and were identified as fengycin, one spot at R*f* 0.6 was identified as iturin A, and the highest spot with R*f* 0.75 was identified as surfactin (Arrebola et al., 2010). Romero et al. (2007) has reported that antifungal effect of cell-free supernatants as well as the presence of the antifungal compounds bacillomycin, fengycin, iturin A, and surfactin are the key factors in antagonism of *B. subtilis* towards *Podosphaera fusca* causing powdery mildew of cucurbits.

### *In vitro* assay of crude antibiotics

The effect of crude antibiotics on the root rot and wilt pathogens of chickpea was tested by agar well diffusion method. The crude antibiotics, at 50 µl volume inhibited the mycelial growth of *F. o*. f. sp. *ciceri* by 41.6% and *R. bataticola* by 40% (Figure 2). The members of the iturin family exhibit strong antifungal and haemolytic activities (Maget Dana and Peypoux, 1994). Fengycin shows specific antifungal activity against filamentous fungi and inhibits phospholipase A2 activity (Nishikiori et al., 1986). When the induction of morphological changes was assayed using crude antibiotics, bulb formation in the mycelia of *F. o*. f. sp. c*iceri* was observed after 6 h of growth, indicating production of antifungal compounds (Figure 3). Although there was germination of conidia, the germ tube formed was abnormal. Light microscopic examination of germinating spores and hyphal tips revealed shrunken, granulated and vesicular cytoplasm as compared to the hyaline, healthy cytoplasm of control hyphae. This was in concurrence with the studies conducted by Tendulkar et al. (2007) on the effect of *Bacillus licheniformis* extracts on *Magnaporthe grisea*.

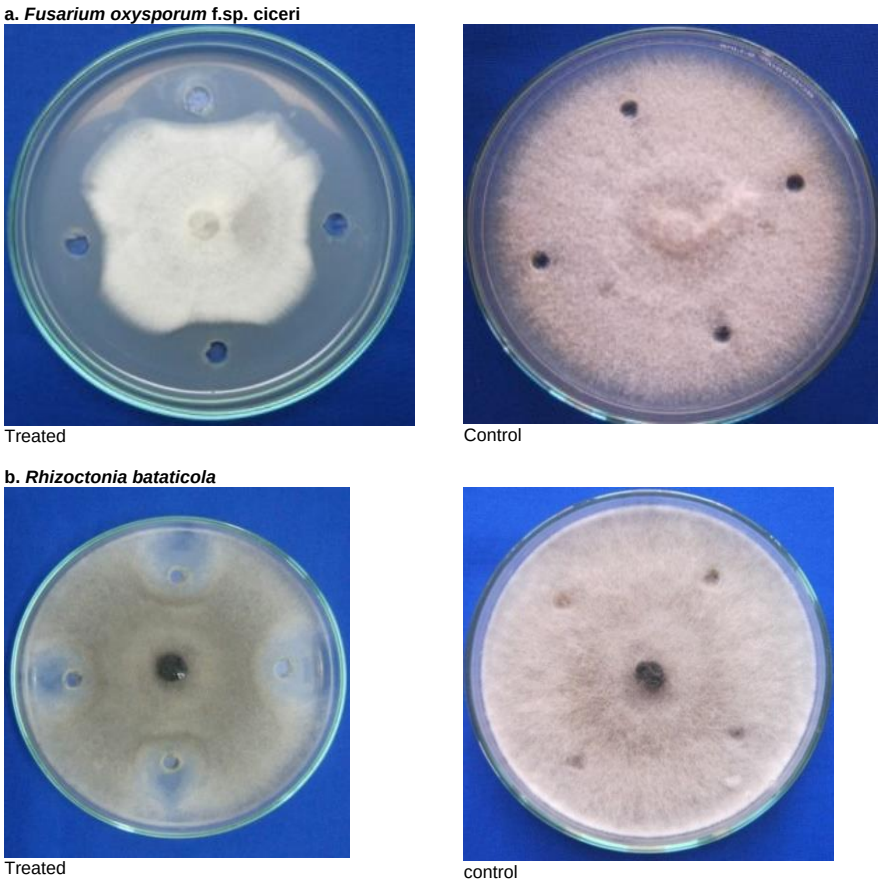


**Figure 2.** Agar well diffusion assay of crude antibiotics of the *Bacillus* strain CaB5.

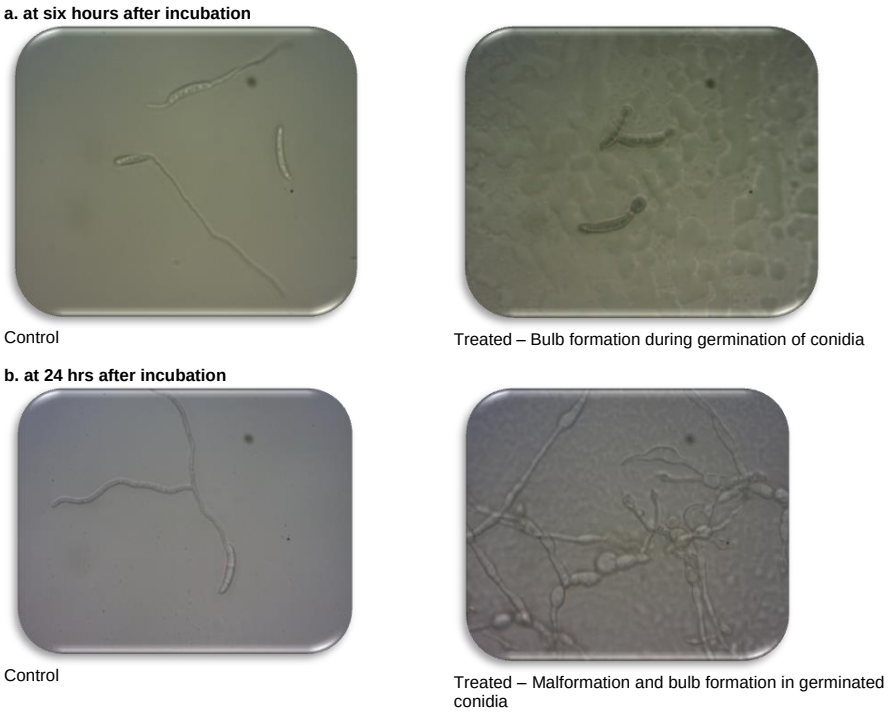


**Figure 3.** Spore germination inhibition assay of *F. oxysporum* f.sp. *ciceri* by cavity slide technique.

## Conclusion

The use of beneficial microorganisms is considered as one of the most promising methods for more rational and safe crop management practices. Based on the results of the study, it is concluded that native *Bacillus* strains efficiently inhibited the growth of root rot (*R. bataticola)* and wilt (*F.o*. f.sp. *ciceri)* pathogens on chickpea by producing an array of lipopeptides like surfactin, iturin and fengycin and also enhanced the seedling vigour of chickpea. Hence *B. subtilis* strain CaB5 can be considered as a promising biocontrol agent for the management of root rot and wilt diseases of chickpea.

## Conflict of interests

The authors did not declare any conflict of interest.

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