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Isolation and characterization of native bacterial antagonists from chickpea rhizosphere and their effect on disease suppression of fusarium wilt

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Abstract
Chickpea (*Cicer arietinum* L.) is a prominent Rabi pulse crop in India. The Indian subcontinent accounts for 90% of total global chickpea production. Fusarium wilt, caused by *F. oxysporum* f.sp. *ciceri*, is the most significant limiting factor in chickpea production, inflicting 10-90% yield losses annually. Hence bacterial antagonists were isolated from the chickpea rhizosphere. Twenty bacterial antagonists were isolated successfully on the basal culture medium Nutrient agar and purified. The cultural and morphological characteristics of 20 bacterial antagonists were studied. Pseudomonas isolates were creamy white to greenish yellow in colour, circular in shape, smooth to glistening edge, butyrous in texture and convex elevation. In case of Bacillus isolates, colonies were fuzzy white in colour, circular lobate to irregular in shape, irregular edge, wrinkled in texture and flat elevation. The biochemical tests of Pseudomonas isolates were positive to Gelatin liquefaction, Catalase, KOH, Oxidase, Fluorescent pigment, Citrate, H2S tests and negative to grams staining and starch hydrolysis. In case of Bacillus isolates, colonies were positive to Gram's staining, Starch hydrolysis, Gelatin liquefaction, Catalase, Oxidase, Citrate and negative to KOH, Fluorescent pigment and H2S tests. Among native and available bacterial antagonists, Bacillus, isolate BRSN-B2 proved best with 64.07% growth inhibition followed by Pseudomonas isolate CRSN-PF1 (48.74%).

Keywords: Chickpea, fusarium wilt, pseudomonas, bacillus & antagonists

1. Introduction
India is the largest chickpea producer as well as consumer in the world. Its cultivation is mainly concentrated in semi-arid environments. It is the most important pulse crop of India and accounts for approximately 75% of world's chickpea production. Major losses in chickpea yield are attributed mainly due to soil borne pathogens. Among the soil borne fungal diseases, Fusarium wilt is one of the most important and devastating disease. The management of plant pathogens in soil with fungicides has been creating problems of fungi resistance, ecosystem imbalance by toxic effects of residues and human and animal health hazards (Mahmood *et al*., 2016) [9]. These problems can be effectively answered by the use of antagonistic microorganisms as biocontrol agents for combating plant diseases (Cook and Baker, 1983; Shamim *et al*., 1997; Ursula *et al*., 2000; Ramadan *et al*., 2016) [4, 13, 15, 11]. Plant growth-promoting rhizobacteria (PGPR) in the rhizosphere have the ability to improve plant growth by colonizing the root system and pre-empting the establishment of and suppressing deleterious microorganisms (Glick, 1996; Kloepper, 2003; Verma *et al.*, 2010; Beneduzi *et al*., 2012) [5, 8, 16, 2]. Among PGPRs, most common are *Pseudomonas fluorescens*, *Bacillus*, *Azotobacter*, *Acinetobacter* and *Rhizobium* spp., etc. During the last decade much of the research has focused on microorganisms belonging to *Bacillus* and *Pseudomonas* species.

2. Materials and Methods
2.1 Isolation and maintenance of bacterial antagonists
Bacterial antagonists were isolated by following serial dilution technique. Ten grams of rhizosphere soil was transferred aseptically into a 250 ml conical flask, containing 90 ml of sterile distilled water and the contents were mixed properly by shaking for five minutes. One ml of aliquot was drawn and transferred to 9.0 ml water blank (containing sterile distilled water). The suspension was shaken for one minute, before it was further diluted. Further, dilution of 10^{-3} to 10^{-7} were obtained and used for isolation of bio-agents. Twenty ml of molten (40 °C) nutrient agar (NA) medium was poured in series of Petri dishes. 0.1 ml of suspension

from respective dilution was transferred aseptically into a Petri dishes containing the medium separately. The suspension was spread uniformly by adopting spread plate method and allowed to solidify. The plates were incubated at 28 ±1 °C and observed at frequent intervals for the development of colonies. Morphologically different colonies appearing on the plates were purified in the NA medium. The purified isolates were preserved at 4 °C. Totally twenty representatives bacterial antagonists were isolated.

2.2. Characterization of bacterial antagonists

Bacterial antagonist isolates were identified according to the colour of the colonies, gram staining and standard biochemical reactions *viz*., Starch hydrolysis, Gelatin liquefaction, Fluorescent pigment, Catalase test, Oxidase test, Citrate utilization test, H_2S test and KOH test were conducted according to Bergey's Manual for Determinative Bacteriology.

2.2.1 Gram staining

A loopful bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat fixed. The smear was flooded for one min. with ammonium oxylate crystal violet. Excess strain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min. decolourized with 95% ethyl alcohol. The smear was washed in gentle stream of water and counter stained with safranin for 30 seconds. The Gram-negative cells appeared red in colour and Gram's positive cells appeared violet in colour.

2.2.2 Potassium hydroxide (KOH) Test

Pure cultures were grown on nutrient agar medium to get loop full mass of colony. A loop-full mass of pure culture was taken on glass slide and mixed with three percent KOH by loop. Formation of string with the loop was recorded as positive for Gram's reaction.

2.2.3 Starch hydrolysis

The 24-hour old cultures were spotted on the starch agar plates and incubated at 28±2 °C for 24 h. After incubation, the plates were flooded with Lugol's iodine solution. Formation of clear zone around the colony was taken as positive for the test (Eckford, 1927).

2.2.4 Catalase test

The test culture was grown on nutrient agar medium (Annexure-I) slants and incubated at 28±20 °C for 24 hours. A loop full of each culture was taken and kept on the slide. Then a few drops of 3% H_2O_2 was poured on the slides over sculture growth and observed for the emission of the effervescence. A positive catalase reaction was indicated by the emission of the effervescence due to production of oxygen as a result of H_2O_2 breakdown (Schaad, 1992).

2.2.5 Oxidase test

An inoculating loop or toothpick was taken. A well isolated colony was touched and spread on an oxidase disc (Disk contains N, N-dimethyl-p-phenylenediamine oxalate and a-naphthol). The reaction was observed within two minutes at 25-30 °C, deep purple blue indicated positive reaction.

2.2.6 Gelatin liquefaction

To the pre-sterilized nutrient gelatin deep tubes, the test cultures were inoculated and tubes were incubated at 28±2 °C for 24 h. The tubes were kept in a refrigerator at 4 °C for 30 minutes. The tubes with cultures that remained liquefied were taken as positive and those that solidified on refrigeration were taken as negative for the test (Blazevic and Ederer, 1975) [3].

2.2.7 Citrate utilization test

Prepared Simmon's citrate medium (a modification of Koser's medium with agar and an indicator) and dispensed in test tubes. Sterilized medium slants at 121 °C for 15 minute and allowed setting of the slants and inoculated these slants with test culturess. To minimize nutrient carryover, the cells were rinsed in distilled water before their transfer to Simmon's citrate agar slants. The slants were incubated for 96 hours at 28±2 °C. A positive test showed a blue colour on the streak of growth. Retention of original green colour and no growth on the line of streak indicated a negative reaction.

2.2.8 Hydrogen sulphide production

Pre-sterilized tubes containing SIM agar (Sulfide, Indole, Motility) were stabbed with the test cultures all along the walls of the test tubes. Inoculated tubes were incubated for 48 h at 28±2 °C. After incubation, the development of black colour along the line of the stab was noted as positive for the test (Cappuccino and Sherman, 1992).

2.2.9 Fluorescent pigment

The petri plates containing sterilized Kings B medium were inoculated with the isolate of *Pseudomonas* sp. incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (365 nm) indicated positive results.

2.3 *In vitro* bio-efficacy of bacterial antagonists against *Fusarium* wilt of chickpea

The antagonistic activity of isolated rhizospheric bacterial antagonists against Fusarium wilt of chickpea was determined by dual culture.

2.3.1 Dual culture

Mycelial disc of 5 mm diameter cut from the margin of 5 days old culture of test pathogen and streak of bacterial antagonists were placed opposite to each other on PDA in Petri plates (90 mm). The petri plates with disc of *Fusarium* alone were served as the control. The inoculated petri plates were incubated at 27±2 °C in BOD incubator. After the completion of incubation period the growth of *Fusarium oxysporum* f. sp. *ciceri* was measured and the% growth inhibition of intersecting colonies was calculated.

3. Result and discussion

3.1 Rhizospheric soil sample collection

To access the rhizospheric bacterial antagonists in chickpea, rhizospheric soil was collected randomly from the wilt infected and healthy chickpea plants from three location *viz*. Cotton Research Station, farm Nanded, Banana Research Station, Nanded and Cotton Research Station, farm Dhanegaon.

Table 1: Details of soil samples collected from chickpea rhizosphere

Sl. No.	Site name	Type	No. of samples collected	Symbolic designation
1.	Cotton Research Station, Farm Nanded	Infected	5	CRSI
		Non-infected	5	CRSN
2.	Banana Research Station, Nanded	Infected	5	BRSI
		Non-infected	5	BRSN
3.	Cotton Research Station, Farm Dhanegaon	Infected	5	CRSDI
		Non-infected	5	CRSDN

3.1.2 Isolation of antagonist bacteria

Bacterial antagonists were isolated by following serial dilution technique. Rhizospheric soil samples were collected from the healthy and infected chickpea plants. Isolated 20 antagonistic bacteria from the rhizospheric soil. Based on the colony and morphological characteristics the isolated bacterial antagonists were designated based on location of the soil sample collected and antagonist.

The observations of present studies are in consonance with the report of earlier worker. Kapali *et al.,* (2016) [7] isolated four isolates of *Bacillus subtilis* (and *Pseudomonas fluorescens* from the rhizosphere soil of chickpea, pigeon pea and citrus. Also, Joseph *et al.,* (2007) [6] collected rhizospheric soil from Chickpea (*C. arietinum* L.) fields and on the basis of morphological, cultural and biochemical characteristics a total of 150 soil isolates were grouped into *Bacillus*, *Pseudomonas*, *Azotobacter*, and *Rhizobium*.

3.2 Cultural characteristic of bacterial antagonists

Shape, colour (also known as pigmentation), texture, elevation and edge (margin) of the bacterial colony was observed under microscope.

3.2.1 Cultural characteristics of Pseudomonas isolates

Among the isolated *Pseudomonas* spp., most of the isolates were creamy white to greenish yellow colonies and circular in shape, smooth edges with butyrous texture and convex in elevation.

The observations of present studies are in full agreement with the report of earlier worker. Mishra *et al*. (2020) [10] examined characteristics including growth, shape and colony appearance on King's B medium, greenish yellow colour was highly appeared around the cream colony with smooth edge, convex, glistening, entire, circular and fluorescent under the sun light that was wider as compare to colony grown on nutrient agar. Similarly, Kapali *et al.,* (2016) [7] isolated four isolates of *Pseudomonas fluorescens* and all the isolates of *P. fluorescens* showed gram negative, transparent, smooth and small colonies with diffusible white and yellow green pigment on King's B medium and all are gram negative rods.

Table 2: Cultural & morphological characters of native *Pseudomonas* isolates

Sl. No.	*Pseudomonas* isolates	Colour	Shape	Edge	Texture	Elevation
1	CRSI-PF1	Creamy white	Circular	Smooth	Butyrous	Convex
2	CRSI-PF2	White	Circular	Smooth	Butyrous	Convex
3	CRSN-PF1	Greenish yellow	Circular	Glistening	Butyrous	Convex
4	CRSN-PF2	Pale yellow	Circular	Glistening	Butyrous	Convex
5	BRSI-PF1	Creamy white	Circular	Smooth	Butyrous	Convex
6	BRSN-PF1	Creamish white	Circular	Smooth	Butyrous	Convex
7	BRSN-PF2	Pale yellow	Circular	Glistening	Butyrous	Convex
8	CRSDI-PF1	Greenish yellow	Circular	Glistening	Butyrous	Convex
9	CRSDI-PF2	Creamish white	Circular	Smooth	Butyrous	Convex
10	CRSDN-PF1	Pale yellow	Circular	Glistening	Butyrous	Convex
11	CRSDN-PF2	Creamish white	Circular	Smooth	Butyrous	Convex

3.2.2 Cultural characteristics of *Bacillus* isolates

Among the isolated *Bacillus* spp., most of the isolate's colonies were white in colour, irregular in shape, flat elevation and irregular edge with wrinkled texture.

The observations of present studies are in full agreement with the report of earlier worker. Tariq *et al.,* (2016) [14] reported that, *Bacillus subtilis* isolates showed slightly raised, flat, regular, irregular, white-, and cream-coloured colonies. Aswathi *et al.,* (2016) [1] reported that, *B. subtilis* were gram positive, rod shaped, white colour and flat colony.

Table 3: Cultural & morphological characters of isolated native *Bacillus* isolates

Sl. No.	Bacillus isolates	Shape	Colour	Edge	Texture	Elevation
1	CRSI-B1	Irregular	Fuzzy white	Irregular	Wrinkled	Flat
2	CRSN-B1	Circular lobate	white	Irregular	Wrinkled	Flat
3	CRSN-B2	Irregular	Grey white	Irregular	Wrinkled	Flat
4	BRSI-B1	Irregular	white	Irregular	Wrinkled	Flat
5	BRSN-B1	Circular lobate	Grey white	Irregular	Wrinkled	Flat
6	BRSN-B2	Circular lobate	white	Irregular	Wrinkled	Flat
7	CRSDI-B1	Irregular	Fuzzy white	Irregular	Wrinkled	Flat
8	CRSDN-B1	Circular lobate	white	Irregular	Wrinkled	Flat
9	CRSDN-B2	Irregular	Fuzzy white	Irregular	Wrinkled	Flat

3.3 Biochemical characteristics of bacterial antagonists

For the identification of bacterial antagonists, certain biochemical tests such as Starch hydrolysis, gelatin liquefaction, fluorescent pigment, catalase test, oxidase test, citrate utilization test, H_2S test and KOH test were conducted according to Bergey's Manual for Determinative Bacteriology.

Among isolated bacterial antagonist's *Pseudomonas fluorescence* was gram negative and showed positive reaction to gelatin liquefaction, catalase, fluorescent pigment, oxidase, KOH, citrate utilization and H_2S tests and among isolated bacterial antagonist's *Bacillus* spp. was gram positive and showed positive reaction to starch hydrolysis, gelatin liquefaction, oxidase and citrate utilization tests.

The observations of present studies are in consonance with the report of earlier worker. Aswathi *et al.*, (2016) [1] reported that, *P. fluorescens* were gram negative, rod shaped and yellow colonies on kings B media. Positive reaction for gelatin liquefaction, starch hydrolysis, oxidase, citrate and catalase tests. All the isolates of *B. subtilis* were gram positive, rod shaped, white colour flat colony and positive reaction for gelatin liquefaction, starch hydrolysis, catalase tests and negative for citrate utilization test. Similarly, Kapali *et al.*, (2016) [7] showed that *P. fluorescens* showed gram –ve, transparent, smooth and small colonies with diffusible white and yellow green pigment on King's B medium and all are gram negative rods. The isolates were oxidase, catalase and citrate positive and indole, Voges-proskauers, methyl red negative. The *B. subtliis* was gram positive, rod shaped and showed profuse growth on NA medium with typical colony morphology which was predominantly off-white to creamish in colour and were indole, methyl red, voges-proskaurs, HCN and IAA negative and oxidase, catalase, citrate positive.

Table 4: Biochemical characterization of isolated bacterial antagonists

Sl. No.	Isolates	GS	SH	GL	CAT	KOH	OXT	FP	CUT	H_2S
1	CRSI-PF1	-	-	+	+	+	+	+	+	+
2	CRSI-PF2	-	-	+	+	+	+	+	+	+
3	CRSI-B1	+	+	+	+	-	+	-	+	-
4	CRSN-PF1	-	-	+	+	+	+	+	+	+
5	CRSN-PF2	-	-	+	+	+	+	+	+	+
6	CRSN-B1	+	+	+	+	-	+	-	-	-
7	CRSN-B2	+	+	+	+	-	+	-	+	-
8	BRSI-PF1	-	-	+	+	+	+	+	+	+
9	BRSI-B1	+	+	+	+	-	+	-	+	-
10	BRSN-PF1	-	-	-	+	+	+	+	+	+
11	BRSN-PF2	-	-	+	+	+	+	+	-	+
12	BRSN-B1	+	+	+	+	-	+	-	+	-
13	BRSN-B2	+	+	+	+	-	+	-	-	-
14	CRSDI-PF1	-	-	+	+	+	+	+	+	+
15	CRSDI-PF2	-	-	+	+	+	+	+	+	+
16	CRSDI-B1	+	+	+	+	-	+	-	+	-
17	CRSDN-PF1	-	-	+	+	+	+	+	+	+
18	CRSDN-PF2	-	-	-	+	+	+	+	+	+
19	CRSDN-B1	+	+	+	+	-	+	-	+	-
20	CRSDN-B2	+	+	+	+	-	+	-	-	-

*GS: Gram Staining SH: Starch Hydrolysis
GL: Gelatin Liquefaction CAT: Catalase test
KOH: Potassium hydroxide Test OXT: Oxidase Test
FP: Fluorescent pigmentation Test CUT: Citrate utilization Test
H_2S: Hydrogen sulphide Test

3.4 *In vitro* bio-efficacy of native and available bacterial antagonists against *Fusarium* wilt of chickpea

A total of six native and available bacterial antagonist isolates CRSN-PF1, BRSI-PF1, BRSN-B2, BRSI-B1, *Pseudomonas fluorescens* (Dept. isolate) and *Bacillus subtilis* (Dept. isolate) were evaluated *in vitro* against *F. oxysporum* f.sp. *ciceri* which exhibited a wide range of mycelial growth and inhibition of the test pathogen.

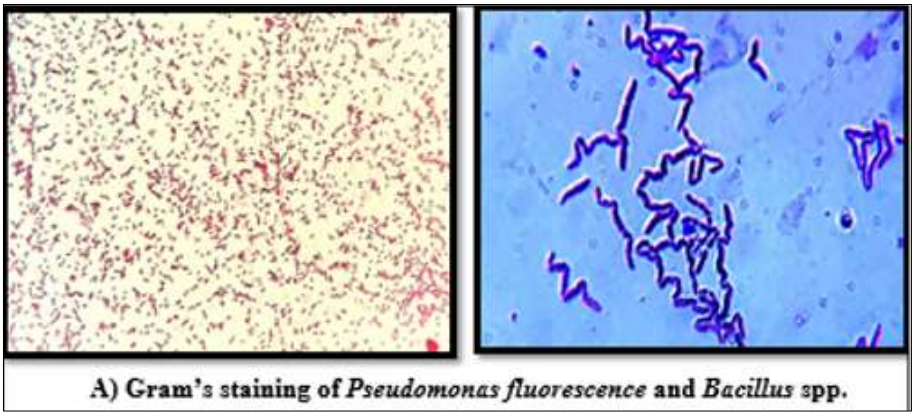
3.4.1 Radial Mycelial growth

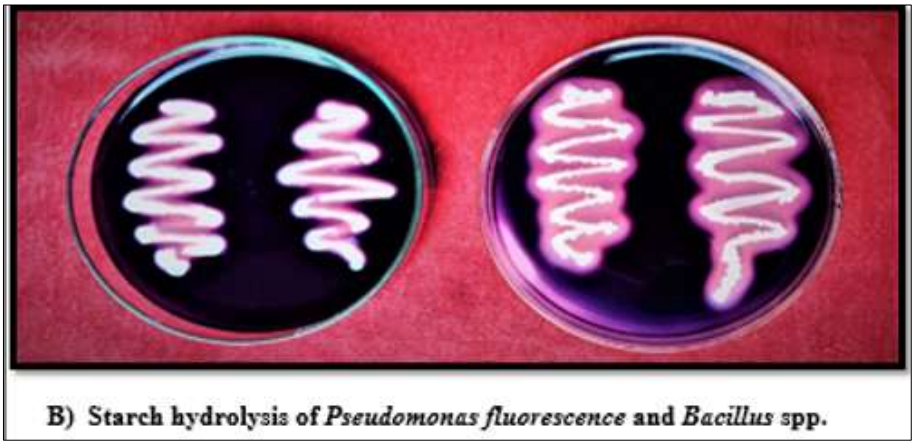
All the tested bacterial antagonist isolates were inhibited a wide range of radial mycelial growth of *F. oxysporum* f.sp. *ciceri* over untreated control (90.00 mm).

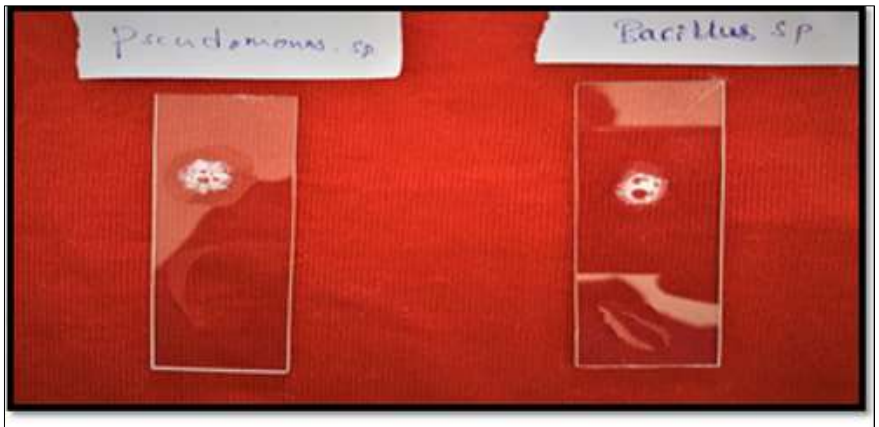
The radial mycelial growth exhibited by the test pathogen (*F. oxysporum* f.sp. *ciceri*) from 32.33 mm (BRSN-B2) to 72.17 mm (BRSI-PF1), growth as against 90.00 mm in untreated control. However, BRSN-B2 was found best with least mycelial growth (32.33 mm) followed by *P. fluorescens* (Dept. isolate) (43.23mm). This treatment followed by CRSN-PF1 (46.13mm), *B. subtilis* (Dept. isolate) (48.67mm), and BRSI-B1 (54.83 mm). Amongst all the tested native bacterial antagonist isolates, BRSI-PF1 was found comparatively less effective with maximum mycelial growth of 72.17 mm.

Plate 1: Pure culture and slants of native *Pseudomonas* (A) and *Bacillus* (B) isolates

Plate 2: Cultural characteristics and Colony morphology of *Pseudomonas fluorescence* (A) and *Bacillus* spp. (B)

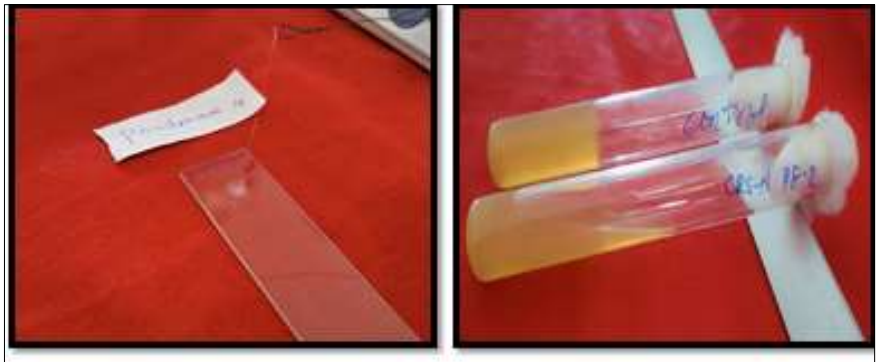






C) Catalase Test

Plate 3: Gram's staining (A), Starch hydrolysis (B) and Catalase tests (C) of *Pseudomonas fluorescence* and *Bacillus* spp.



A) KOH and Gelatin liquefaction Test

B) H_2S and Oxidase Test

C) Fluorescent pigment and citrate utilization Test

Plate 4: KOH and Gelatin liquefaction (A), H_2S and Oxidase (B) and fluorescent pigment and citrate utilization (C) Tests of bacterial antagonists

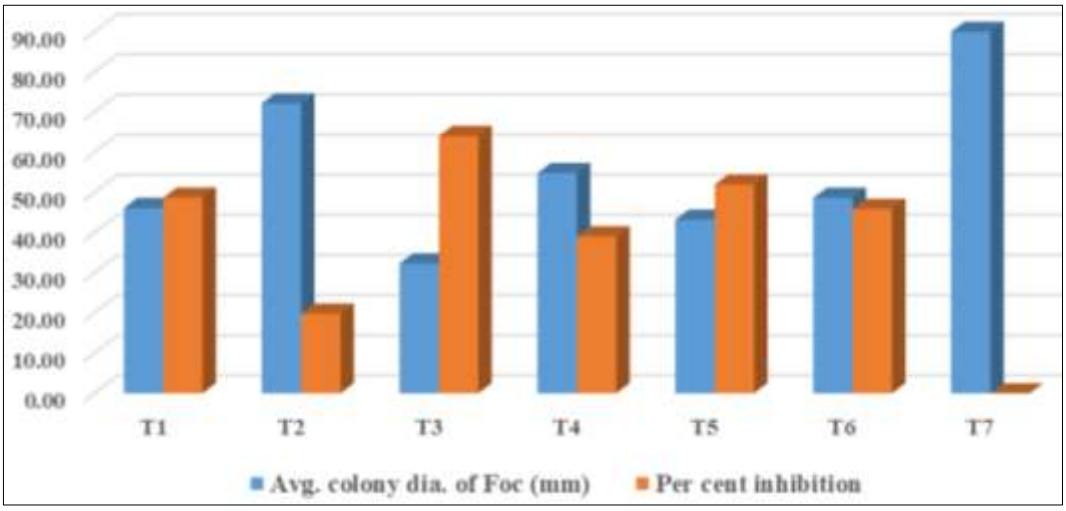
Plate 5: Dual culture of native and available bacterial antagonists against *Fusarium oxysporum* f.sp. *ciceri*

3.4.2 Mycelial growth inhibition

All the tested bacterial antagonist isolates tested were significantly inhibited mycelial growth of *F. oxysporum* f.sp. *ciceri* over untreated control.

% mycelial growth inhibition of the test pathogen (*F. oxysporum* f.sp. *ciceri*) was ranged from 19.81% (BRSI-PF1) to 64.07% (BRSN-B2). However, BRSN-B2 was found best with highest mycelial growth inhibition (64.07%) followed by *P. fluorescens* (Dept. isolate (51.96%). This treatment followed by CRSN-PF1 (48.74%), *B. subtilis* (Dept. isolate) (45.93%), and BRSI-B1 (39.07%). Amongst all the tested native bacterial antagonist isolates, BRSI-PF1 was found comparatively less effective with least mycelial growth inhibition of 19.81%.

Similar *in vitro* mycelial inhibition effects of bacterial antagonists against *F. oxysporum* f.sp. *ciceri* infecting chickpea was observed by Kapali *et al*., (2016) [7] revealed that the radial mycelial growth of Foc with *P. fluorescens* was ranging from 35.66 to 81.33mm and percent inhibition ranging from 9.63 to 60.37% and radial mycelial growth of Foc with *B. subtilis* was ranging from 28.33 to 78.87mm and percent inhibition ranging from 12.36 to 68.52%. Similarly, Rani and Mane (2014) [12] accessed the radial mycelial growth and percent inhibition of Foc with *P. fluorescens* was 41.83mm and 53.52% and radial mycelial growth and percent inhibition of Foc with *B. subtilis* was 33.17 mm and 63.14%.



T_1: CRSN-PF1
T_2: BRSI-PF1
T_3: BRSN-B2
T_4: BRSI-B1
T_5: *Pseudomonas fluorescens* (Dept. isolate)
T_6: *Bacillus subtilis* (Dept. isolate)
T_7: Control

Fig 1: *In vitro* bio-efficacy of native and available bacterial antagonists against *Fusarium* wilt of chickpea

Table 5: *In vitro* bio-efficacy of native and available bacterial antagonists against *Fusarium* wilt of chickpea

T	Isolates	Colony Dia.* (mm)	% inhibition of mycelia growth
T_1	CRSN-PF1	46.13	48.74 (44.28)#
T_2	BRSI-PF1	72.17	19.81 (26.43)
T_3	BRSN-B2	32.33	64.07 (53.17)
T_4	BRSI-B1	54.83	39.07 (38.69)
T_5	*Pseudomonas fluorescens* (Dept. isolate)	43.23	51.96 (46.13)
T_6	*Bacillus subtilis* (Dept. isolate)	48.67	45.93 (42.66)
T_7	Control	90.00	0.00 (0.00)
SE±		0.49	0.31
CD @ 1%		2.05	1.32

*Mean of three replications, Dia. =Diameter,
(# Figures in parenthesis are arc sine transformed value)

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