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Microbiome characterization of PGPR isolated from *Fusarium* wilt infected cumin Rhizosphere

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Abstract
The plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria which actively colonize plant root and benefit plants by providing growth promotion. The present study reports the analyses of the rhizospheric microbiome of the cumin (*Cuminum cyminum* L.) interaction with *Fusarium* wilt. Total 164 isolates were obtained from 10 healthy and *Fusarium oxysporum* f. sp. *Cumini* (*FoCu*) wilt infected rhizosphere under *in-vitro* condition. After haemolysis test, 60 isolates were found non-pathogenic and further characterised for PGPR and antagonist activity against *FoCu*. The 45 isolates were positive for IAA production, six isolates were able to ACC (1-aminocyclopropane-1- carboxylic acid) activity, fiveisolates were able solubilise phosphate, eight isolates were able to hydrolysis of chitin and eleven isolates have zinc solubilizing ability under *in vitro*. The H1-2 and H1-23 isolates were considered as *Bacillus subtilus* and found with potent antagonist activity. These two bacterial isolates H2 (39.47%) and H23 (52.63%) were suppressed the growth of *FoCu*. Potent *Bacillus* isolates derived from microbiome characterisation may be useful as PGPR and antagonist as dual purpose for betterment of cumin cultivation.

Keywords: Microbiome, PGPR, *Fusarium* wilt, antagonism, SEM interaction

Introduction
Cumin (*Cuminum cyminum* L.) locally known as "Jeera" belongs to family Apiaceae. The demand for cumin is increasing in domestic as well as worldwide market which performs a critical function in national financial system. However, the production and productivity of cumin is decreasing year after year due to several reasons. One of them heavy infestation of diseases and pests. Number of microorganism present in soil rhizosphere may be affected by cultivation methods, climate, soil nutrient application, insecticide, and pesticide. Soil management practices has adverse effects on beneficial organisms. The cumin and other crops in India often suffer from wilt diseases caused by fungi. Cumin wilt caused by *F. oxysporum* f. sp. *cumini* has been noticed 35 per cent yield losses in some district of Rajasthan (Vyas and Mathur, 2002) [57].

In field condition the infected plants first show changes in colour of leaves from green to yellow, beginning from oldest leaves and extending upward to the younger leaves leading to wilting of the entire plant which ultimately dries up and could easily be pulled out from soil (Gour and Agrawal, 1988) [19]. The rhizosphere zone around the root is the largest microorganism's ecology in the world. The rhizomicrobiome has the capability to help fight numerous biotic and abiotic stresses as well as enhance plant health and productivity.

Another important feature of the bacteria is the direct growth plant promotion phenomenon. The bacteria known as plant growth-promoting rhizobacteria (PGPR) live in close vicinity to the plants (endosphere or rhizosphere) and play a key role in the transformation of many organic and inorganic compounds making them available for plant growth such as phosphorus and zinc (Olanrewaju *et al*., 2017) [39]. Furthermore, these PGPR exert beneficial effects on plant growth and yield by the production of plant growth regulating substances, such as indole-3-acetic acid (IAA) and acid, cytokinin and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick, 2014) [18]. The ACC-deaminase producing PGPR facilitate plant growth by decreasing plant ethylene levels and reducing stress caused by biotic (such as phytopathogenic bacteria/fungi) and abiotic factors (such as heavy metals, salt, and drought) (Glick *et al*., 2007; Shahid *et al*., 2012) [17, 48].

Biofertilization, phytostimulation and biological control are diverse traits of heterogeneous PGPR and can be exploited to develop formulations to control pathogens, increase yield and food production by using fewer resources and less reliance on the chemical fertilizers and pesticides (Bhattacharyya and Jha, 2012) [4].

Because of the changing climates, high prices of agrochemicals, and ecological crises, we hypothesize that devising multi-purpose bioformulations will be a more practical strategy for integrated pest and nutrient management. This study aimed to identify potential rhizobacterial strains exhibiting plant growth promotion as well as biocontrol potential against a broad spectrum of phytopathogenic fungi. The objective is to develop a dual-purpose inoculum exhibiting bio-fertilizer and biopesticide potential for commercial use in integrated nutrient management and sustainable agriculture.

Material and Methods

Sample collection and isolation of bacteria

Cumin (GC-4) healthy and *FoCu* infected rhizosphere soil samples were collected from different five location like Junagadh (Pathology field), Khadiya, Patapur, Limadhra, Junagadh (Entomology field) as described Somasegaran and Hoben, 1994 [54]. Approximately one gram of soil was taken and appropriate dilutions 10^{-3} to 10^{-7} were used to inoculate on nutrient agar (NA) plates. After 24 h of inoculation each colony was selected and transferred on N-agar media.

Morphological characterization

The Colony morphology *viz*., size, color, shape and growth pattern were recorded after 48 h of growth on NAplates at 27 ± 2 °C as described by Somasegaran and Hoben, 1994 [54]. Cell size and motility were observed by light microscopy. The isolated bacteria were characterized for morphological by gram staining test.

Qualitative biochemical characterization

Urease test

The christensen's urea agar medium (e ne- 1g/L, dextrose - 1g/L, sodium chloride- 5g/L, potassium phosphate monobasic- 2g/L, phenol red- 0.012g/L, agar- 15g/L) was prepared and sterilized in autoclave at appropriate parameters. After sterilization, the medium was cooled to palm bearable temperature and 20g/L of urea was added. Mixed well, inoculated and incubated at room temperature for four days. The results were observed. Urease test helps in the identification of microorganisms having ability to produce urease enzyme. This enzyme belongs to the amidohydrolases and phosphodoesterases super families. Urease causes the hydrolysis of urea into NH3 and carbon dioxide. The ammonia formation was changed the pH of the medium to alkaline and colourmay also change to pink at pH 8.1 indicating positive results (Bailey and Scott, 1974) [2].

Starch utilization test

Starch Agar Medium (starch – 2g/L, peptone - 5g/L, beef extract – 3g/L, agar – 30g/L) was prepared and sterilized. The single streak inoculation technique was adopted; the sample organism was streaked on the plate and incubated for 24 hrs at 37°C. After incubation the plates were flooded with Gram's iodine solution and observed for clear zone. To convert starch into small molecules release enzymes that hydrolyse the starch into glucose that may enter into metabolic pathways and used as energy source. To check this iodine was added to the agar medium that may turn to dark brown colour due to hydrolysis of starch indicating positive results (Hemraj *et al*. 2013).

Nitrate test

For nitrate test, add sulfanilic acid (also represented as nitrate I) and dimethyl-alpha-napthalamine (also represented as nitrate II). The presence of nitrite in the media, if the nitrite was present in the medium, it may react with both reagents I and II. Due to this reaction red colour formation indicated positive result while no red colour formation indicated negative result (MacFaddin, 2000) [34].

Methyl red test

The methyl red broth (peptone -7g/L, glucose- 5g/L, potassium phosphate- 5g/L) was autoclaved at 15lb/inch² for 15mins, inoculated with sample and incubated at 37°C for 48hrs. After incubation five drops of methyl red indicator were added and observed for colour change. Acid production decreases the medium pH that changes the colour of the methyl red from yellow to red indicate the potential of bacteria touse glucose present in the culture medium (Crown and Gen, 1998) [14].

Gelatine oxidase test

Gelatine is composed of short amino acid polymers and their derivatives. They were considered as nitrogen and carbon source for a wide variety of microbes. Nutrient gelatine agar medium (gelatine- 120g/L, beef extract- 3g/L, peptone-5g/L, agar-15g/L) were sterilized and inoculated by the sample organism and incubated at 35 °C for ten days. After incubation the tubes were kept in ice to check liquefaction of the medium (Sivanandhini *et al*. 2015) [53].

Motility test

Beef extract (3. 0 g), Pancreatic digest of casein (10. 0 g), Sodium chloride (5. 0 g), Agar (4. 0 g), Bring to 1 L with distilled water were used to prepare media followed by heating for 1 Minute. 5 ml of 1% 2, 3, 5-Triphenyl-2H-Tetrazolium Chloride (TTC) solution were added. Dispense in 5-ml aliquots into tubes and autoclave at 121°C under 15 psi pressure for 15 minutes than cool upright in racks. After inoculation, incubate at 35 °C for 18 hours or until growth was evident (Sivanandhini *et al*. 2015) [53].

Citrate test

The slants were prepared for Simmon's Citrate Agar Medium. By stabbing and streaking method the samples were inoculated in the respective slants and incubated at 37°C for 48 hrs. The results were examined. The change in pH may change the colour of bromothymol blue from green to blue when the pH rise above 7.6. Bromothymol blue was used as indicator in CAU test (Sivanandhini *et al*. 2015) [53].

Quantitative biochemical characterization

IAA production

There are several phytohormone groups and the best known is the auxin group. Indole 3-acetic acid (IAA) production was determined by the method described by Loper and Scroth (1986) [32]. The rhizosphere cultures were grown in nutrient medium amended with 1 mg/ml tryptophan at 28+2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. 2 ml of the supernatant was acidified with 2 drops of 10 mm orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid, 1 ml, 0.5M FeCl3 solution) was mixed to it. Development of pink color indicates presence of IAA in

the supernatant. The optical density was read spectrophotometrically at 530 nm. Concentration of IAA was measured by comparing with the standard graph obtained using various concentrations of standard IAA (Himedia) (Shokri and Emtiazi, 2010) [50].

ACC-deaminase activity

DF (Dworkin and Foster) medium containing in gram. I'Glucose, 0.1; KH_2PO4, 1.36; Na_2HPO4, 2.13; $MgSO4.7H_2O$, 0.2; $CaCl2.2H_2O$, 0.7; $FeSO4.7H_2O$, 0.2; $CuSO4.5H_2O$, 0.04; MnSO4. H_2O, 0.02; $ZnSO4.7H_2O$, 0.02; HBO3, 0.003; $CoCl2.6H_2O$, 0.007; $Na_2MoO4.2H_2O$, 0.004; ACC, 5 mm in distilled water. Bacteria were platted on nitrogen-deficient DF, DF-ACC, and DF-ammonium sulphate media. The DF medium was prepared as described by Penrose and Glick (2003) [40]. ACC stock solution (0.5 M) was filter-sterilized through a 0.2 µm membrane, and stored frozen (20°C). Just prior to use, the frozen ACC solution was thawed and added to autoclaved DF minimal medium obtain the DF-ACC medium with a final ACC concentration of 3 mm. Solid DF, DF-ACC and DF-NH3 media were supplemented with 1.5% (WV^{-1}) agar. Bacterial isolates were streaked on DF, DF-ACC and DF-NH, agar and incubated for 48 h at 28 °C.

Phosphate solubilization ability

The phosphate solubilization capability of the isolated strains of rhizobacteria was determined by qualitative method. The plates were prepared with Pikovaskya's medium. The cultures of isolates were spot inoculated on the plates and incubated in an incubator at 28 °C for 5-7 days. Formation of clear zone around the microbial colonies indicated phosphate solubilisation (Banerjee *et al*., 2010) [3].

Chitinase activity

Chitin hydrolysis was checked by the method of Roberts and Selitrennik off with a few modifications described as follows: spotting isolated bacteria on the center of 1% colloidal chitin agar (CCA) (colloidal chitin-1%. NaNO3-0.2 g, K_2HPO-0.1 g, MgSO4-0.1 g, CaCO3-0.1 g, $FeSO4\ 7H_2O$-0.001 g. KCL-0.05 g) media at neutral pH and were incubated at 30°C. The zone of clearance due to chitin hydrolysis was recorded up to 8 days. The isolates those formed clear zone of hydrolysis above 5 mm were selected (Boller and Mauch, 1988) [5].

Zinc solubilization ability

All bacterial strains were screened for their zinc solubilizing ability for insoluble zinc compound zinc carbonate (ZnCO3). Overnight grown single colonies were transferred aseptically by inoculating as spot on respective zinc medium plates (Sharma *et al*., 2012) [49]. These plates were covered with aluminium foil and incubated in dark at 28 °C for 12 days. Zinc solubilizing strains produced clear zones around colonies. The diameter of these zones was recorded.

Antagonist potential of bacterial isolates against *FoCu*

Isolation of the pathogen (*FoCu*) from infected plant sample

FoCu was isolated from infected plants through tissue isolation technique. The infected root were surface sterilized. They were washed in sterile distilled water. Then they were washed with 0.1% mercuric chloride for 1 min then thoroughly washed in sterile distilled water for 2-3 times. Then the roots were washed in 70% ethanol for 10 sec and immediately rinsed with sterile distilled water for 2-3 times. The infected part of the roots were trimmed to expose the fungus. The trimmed root pieces were washed once in sterile distilled water and placed on the agar surface. The inoculated plates were incubated at 25 °C for 5-7 days. Pureculture of pathogen were derived by subsequent transformation of pure mycelia on potato dextrose agar (PDA) medium. The phytopathogen was identified based on morphology on PDA media (Kumar *et al*. 2016) [30].

***In vitro* screening of antifungal activity by dual plate technique**

Actively growing fungal mycelial plugs were placed in sterile PDA plates in the centre. The rhizosphere isolates were streaked on the edge of the plates. The plates were incubated for 8 days at 28 °C. The antifungal activity of rhizosphere isolates against the fungal pathogen was confirmed by observing the inhibition of the fungal colony on the vicinity of rhizosphere isolates (Kumar *et al*. 2016) [30]. The percent inhibition (PI) of radial growth of fungus was calculated by the formula:

$$PI = \frac{(R-r)\times 100}{R}$$

Where PI = Percent inhibition; R = Radial growth of pathogen in control plate and r = Radial growth of the fungal colony interacting with antagonistic bacteria (Tiwari *et al*. 2016).

SEM characterization of potent antagonist pathogen and their interaction

Microscopic structures were observed using Scanning Electron Microscope (SEM). Briefly, a 1 cm^2 section of 8 days old fungal culture plate was used as specimen. Sample was immersed in 2.5% glutaraldehyde and kept overnight at 4 °C. For dehydration, the specimen was transferred sequentially to 30%, 50%, 70%, 80%, 90%, 100%, and 100% alcohol. The sample was transferred into a 1:2 solution of hexamethyldisilazane (HMDS): 100% Ethenol (EtOH) and kept for 20 min. Fresh solution of 2:1 HMDS: EtOH was added and further transferred into 100% HMDS. Each step was repeated twice for 20 min. After drying overnight, the sample stub coated with gold-palladium followed by mounting on a Carl Zeiss EVO18 Scanning Electron Microscope. The specimen was observed under low vacuum mode for features such as shape of conidial heads, serration, morphology and ornamentation (Lombard *et al*. 2019) [31].

Efficacy of potent bacterial isolates (H1-2 and H1-23) against *Fusarium* wilt under pot culture

Preparation of bacterial inoculation for cumin GC-4 seed treatment

Bacterial strains H1-2 and H1-23 were cultured in 250 ml conical flasks containing 200 ml nutrient broth on shaker at 120 rpm for 48 h at 28 ± 2 °C. Bacterial cells were collected via centrifugation at 15000 rpm for 1 min at 4 °C, and each pellet was washed twice with sterilized distilled water (SDW). The bacterial pellets were suspended in 3 ml SDW, vortexed and used for seed treatment. Approximately 30 cumin seeds were surface sterilized in 5% sodium hypochlorite solution for 1 min and washed three times in SDW. Seeds were treated with five treatments: (i) Cumin seeds soaked in SDW (T1); (ii) Cumin seeds inoculated with 10^6 cfu/mL of the *FoCu*(T2);

(iii) Cumin seeds inoculated with 10^6 cfu/mL of the first dominant bacterial isolate (T3); (iv) Cumin seeds inoculated with 10^6 cfu/mL of the second dominant bacterial isolate (T3) and (iiv) Cumin seeds inoculated with 10^6 cfu/mL of the combination of dominant bacterial isolates. The bacteria-coated seeds were scattered on a petri dish and allowed to soak overnight at room temperature. The excess water is decanted in the morning and the seeds were used for the pot studies (Kumar *et al*., 2016) [30].

Pot trial

In pot experiment, 4 kg soil was transferred to each plastic pot and moistened suitably 24 hr before soil inoculation with the pathogen. The *FoCu* isolates were multiplied on potato dextrose broth in Erlenmeyer flasks which were sterilized at 15 p.s.i for 30 min. These flasks containing the sterilized media were inoculated with respective *FoCu* isolates and incubated at 26 ±1 0C for 12 days. Mycelial suspension of individual *Fusarium* isolate was added to soil at 5 g/kg soil and mixed thoroughly. The harvested fungal mat was macerated and homogenized. The mycelial suspension was prepared by taking around 20 g fresh mycelia in 350 ml sterilized distilled water for each pot and allowed to stabilize for 72 hr before sowing of cumin seeds (Kumar *et al*., 2016) [30].

Assessment of disease incidence

The wilt disease was observed and incidence of disease was calculated by the following formula: Disease incidence = No. of infected plants/Total no. of plant assessed x 100. To assess the disease incidence used as three replications (Waller *et al*., 2002) [58]. In experiment, the data of disease percent was transformed to their Arcsin values (Fisher and Yates 1963) [16]. The statistical analysis of the data of pot trial experiments were done following completely randomized block design. The data of field experiments were analysed following randomized block design (Cochran and Cox 1957) [12].

Results and Discussion

Cumin (GC-4) healthy and *FoCu* infected rhizosphere soil samples were collected from different five location. Total 164 bacterial isolates obtained from ten healthy and infected cumin rhizosphere soil samples (Table 1) on NA agar plates and were subjected to pathogenicity using haemolysis test.

Table 1: Details of no of isolated bacteria derived from different cumin (GC-4) rhizosphere soil on N-agar

Sr. No	Area of collection	Status of cumin rhizosphere	Source ID	Total Isolates obtained	No. of isolates after haemolysis test
01	Junagadh (Pathology field)	Healthy	H1	24	11
		Infected	I1	19	04
02	Khadiya	Healthy	H2	16	03
		Infected	I2	14	04
03	Patapur	Healthy	H3	18	07
		Infected	I3	09	01
04	Limadhra	Healthy	H4	13	05
		Infected	I4	17	09
05	Junagadh (Pathology field)	Healthy	H5	22	14
		Infected	I5	12	02
Total				164	60

(H1= Healthy 1, I1= Infected 1, H2= Healthy 2, I2= Infected 2, H3= Healthy 3, I3= Infected 3, H4= Healthy 4, I4= Infected 4, H5= Healthy 5, I5= Infected 5)

Morphological characterization

After haemolysis test, 60 bacteria were found non-pathogenic and further characterise for gram staining. Total 24 isolates were observed rod shape, 20 isolates stained blue in colour and hence identified as gram positive. Thus we can said all 20 isolates were *Bacillus* spp. The isolated colonies on N-agar plate observed for different shape, colour, colony elevation of colonies and shape of cell (Table 2). Among 60 isolates, 4 were in irregular shape and 56 isolates were round shape. The colonies of eighteen bacterial isolates were creamy white, ten isolates transparent, nine isolates in white, four isolates in transparent white, three isolates in brownish white, two isolates in whitish yellow, another two isolates in whitish red and one isolates examined in light yellow, blackish white, light orange. Fifty eight bacterial colonies were flat in elevation while two were found raised in elevation. Among 60 isolates, the cell shape of twenty four isolates were rod shape, nineteen isolates were round shape, twelve isolates were in cocci shape, four isolates were in short rod shape and one isolates was in curved rod shape (Table 2).

Table 2: Morphological & microscopic characterization of 60 bacterial isolates derived from healthy and wilt infected rhizosphere

Sr. no.	Isolates ID	Colony colour	Colony shape	Colony Elevation	Cell shape	Gram Reaction
1	H1-2	Creamy white	Irregular	Flat	Rod shape	Positive
2	H1-3	White	Round	Flat	Round shape	Positive
3	I1-4	Light Orange	Round	Flat	Rod shape	Positive
4	H1-4	Transparent	Irregular	Flat	Round shape	Positive
5	H1-6	Creamy	Round	Flat	Cocci	Negative
6	I1-6	Transparent	Round	Flat	Rod shape	Positive
7	I1-8	Creamy Orange	Round	Flat	Cocci	Negative
8	H1-9	Creamy	Round	Flat	Rod shape	Positive
9	H1-11	Blackish white	Round	Raised	Round shape	Positive
10	H1-12	Whitish Yellow	Round	Flat	Round shape	Positive
11	H1-15	Whitish Red	Round	Flat	Round shape	Negative
12	I1-15	Transparent	Round	Flat	Rod shape	Positive

13	H1-20	whitish Red	Round	Flat	short round	Positive
14	H1-23	Creamy white	Round	Flat	short round	Positive
15	H1-24	Greyish White	Round	Flat	rod shape	Positive
16	I2-22	White	Round	Flat	Cocci	Positive
17	I2-28	Creamy White	Round	Flat	Rod shape	Positive
18	I2-30	Brown	Round	Flat	Rod shape	Positive
19	H2-31	Creamy white	Round	Flat	Rod shape	Positive
20	I2-31	Pinkish White	Round	Flat	Rod shape	Positive
21	H2-32	White	Round	Flat	Round shape	Positive
22	H2-33	Transparent White	Round	Flat	Short rod shape	Positive
23	I3-37	Creamy White	Round	Flat	Rod shape	Positive
24	H3-42	White	Round	Flat	Round shape	Positive
25	H3-45	Brownish	Round	Flat	Rod shape	Positive
26	H3-48	Whitish Transparent	Round	Flat	Round shape	Positive
27	H3-50	Creamy white	Round	Flat	Curved rod shape	Positive
28	H3-55	Transparent	Round	Flat	Cocci	Positive
29	H3-56	Creamy	Round	Flat	Rod shape	Negative
30	I4-42	Transparent White	Round	Flat	Round shape	Positive
31	I4-43	Creamy White	Round	Flat	Cocci	Positive
32	I4-44	Creamy White	Round	Flat	Cocci	Positive
33	I4-47	White	Round	Flat	Rod shape	Positive
34	I4-49	Transparent Creamy	Round	Flat	Rod shape	Negative
35	I4-50	Transparent	Round	Flat	Rod shape	Positive
36	I4-53	Transparent Pinkish	Round	Flat	Cocci	Positive
37	I4-57	White	Round	Raised	Rod shape	Positive
38	I4-58	Transparent	Round	Flat	Round shape	Positive
39	I5-59	Yellowish White	Round	Flat	Cocci	Positive
40	I5-60	Pinkish Brown	Round	Flat	Rod shape	Positive
41	H4-63	Creamy Yellow	Round	Flat	Round shape	Positive
42	H4-64	Creamy Yellow	Round	Flat	Cocci	Positive
43	H4-65	Pink	Irregular	Flat	Round shape	Positive
44	H4-71	Yellowish	Round	Flat	Cocci	Negative
45	H4-72	Brownish	Round	Flat	rod shape	Negative
46	H5-73	White	Round	Flat	Round shape	Positive
47	H5-75	Transparent	Round	Flat	rod shape	Positive
48	H5-76	Creamy white	Round	Flat	rod shape	Positive
49	H5-77	Creamy white	Round	Flat	Round shape	Positive
50	H5-78	Transparent	Round	Flat	Rod shape	Negative
51	H5-79	Transparent	Round	Flat	Round shape	Positive
52	H5-80	White	Round	Flat	Rod shape	Positive
53	H5-81	Powdery White	Round	Flat	Cocci	Positive
54	H5-82	Creamy white	Round	Flat	Round shape	Positive
55	H5-83	Creamy white	Round	Flat	Round shape	Negative
56	H5-84	Creamy white	Round	Flat	Cocci	Negative
57	H5-86	Orange	Round	Flat	rod shape	Positive
58	H5-92	Transparent	Round	Flat	short rod	Negative
59	H5-94	Creamy white	Round	Flat	Rod shape	Positive

(H1= Healthy 1, I1= Infected 1, H2= Healthy 2, I2= Infected 2, H3= Healthy 3, I3= Infected 3, H4= Healthy 4, I4= Infected 4, H5= Healthy 5, I5= Infected 5)

Qualitative biochemical characterization

Various biochemical tests were carried out. Among 60 isolates, twenty two isolates gave positive result for urease test, forty five isolates gave positive result for starch utilization, sixteen isolates gave positive result in nitrate test, ten isolates gave positive result in methyl red test, thirty one isolates gave positive result in gelatine test, twenty seven isolates gave positive result in motility test and four isolates gave positive result in citrate test (Table 3). It was found that, the bacterial isolates were gram positive spore of *Bacillus* Spp. The VP test for this bacteria was positive, while methyl red tests were negative (Lu *et al*. 2018) [33].

Table 3: Antagonistic and biochemical test of isolated bacteria

Sr. no	Isolates	Antagonistic activity against *Fusarium* wilt	Urease	Starch	Nitrate	MR	Gelatine	Motility	Citrate
1	H1-2	+	-	+	-	-	-	-	-
2	H1-3	-	+	+	+	-	-	-	-
3	I1-4	-	+	+	+	+	+	+	+
4	H1-4	-	-	-	+	-	-	+	-
5	H1-6	-	-	+	-	+	+	+	-
6	I1-6	-	+	+	+	+	+	+	-
7	I1-8	-	+	+	-	-	-	+	-

8	H1-9	-	-	+	+	-	+	-	-
9	H1-11	+	-	+	-	-	+	-	-
10	H1-12	+	+	-	+	-	+	-	-
11	H1-15	-	-	+	-	-	-	+	-
12	I1-15	-	-	+	+	-	+	+	+
13	H1-20	+	+	+	-	-	-	-	-
14	H1-23	+	-	+	-	-	+	-	-
15	H1-24	-	-	+	-	-	+	+	-
16	I2-22	-	+	+	+	-	+	+	+
17	I2-28	+	+	+	+	-	-	+	-
18	I2-30	-	-	-	-	-	-	+	-
19	H2-31	-	+	+	-	-	-	+	-
20	I2-31	-	-	-	-	-	+	-	-
21	H2-32	-	-	-	-	-	-	-	-
22	H2-33	-	+	+	-	-	-	+	-
23	I3-37	+	+	+	-	+	+	-	-
24	H3-42	-	-	-	-	-	+	-	-
25	H3-45	-	-	+	-	-	+	-	-
26	H3-48	-	-	-	-	-	-	-	-
27	H3-50	-	-	-	-	-	-	-	-
28	H3-55	-	-	+	-	-	-	+	-
29	H3-56	-	-	+	_	-	+	-	-
30	I4-42	+	-	+	+	-	+	+	-
31	I4-43	+	+	+	-	-	+	-	-
32	I4-44	-	+	+	-	-	+	+	-
33	I4-47	-	+	+	+	-	+	+	-
34	I4-49	-	+	+	-	-	+	-	-
35	I4-50	-	-	+	+	-	+	-	-
36	I4-51	-	-	-	-	-	-	-	-
37	I4-53	-	-	-	-	-	+	-	-
38	I4-57	-	+	+	-	-	_	+	-
39	I4-58	-	-	-	-	-	+	-	-
40	I5-59	-	-	+	+	+	+	+	+
41	I5-60	-	-	+	-	+	+	-	-
42	H4-63	-	+	+	-	+	-	+	-
43	H4-64	-	-	-	-	-	-	-	-
44	H4-65	-	+	+	-	+	-	-	-
45	H4-71	-	+	-	-	-	-	-	-
46	H4-72	-	-	+	+	+	+	+	-
47	H5-73	-	-	+	-	-	-	-	-
48	H5-75	-	-	-	+	-	+	+	-
49	H5-76	-	+	+	-	-	-	-	-
50	H5-77	-	-	+	-	+	-	-	-
51	H5-78	-	-	+	-	-	-	+	-
52	H5-79	-	-	+	-	-	+	+	-
53	H5-80	-	-	+	-	-	+	+	-
54	H5-81	-	-	+	-	-	+	-	-
55	H5-82	-	+	+	-	-	-	+	-
56	H5-83	-	-	+	+	-	-	+	-
57	H5-84	-	+	+	-	-	-	-	-
58	H5-86	-	-	+	+	-	-	+	-
59	H5-92	-	-	+	-	+	-	-	-
60	H5-94	-	-	+	+	-	-	-	-

(H1= Healthy 1, I1= Infected 1, H2= Healthy 2, I2= Infected 2, H3= Healthy 3, I3= Infected 3, H4= Healthy 4, I4= Infected 4, H5= Healthy 5, I5= Infected 5)

Quantitative biochemical characterization

IAA production

The bacterial isolates were screened for indole acetic acid (IAA) production. Our study showed that the 45 isolates were found to have the ability to produce IAA (Table 4). Among 45 isolates, A 15 isolates were strongly positive. The IAA production by the isolates ranged from 9.04 (H3-48) to 36.15 (H5-80) µg/ml. The amount of IAA was estimated from standard graph. Mary Franceena *et al*. (2016) [36] screened soil bacteria for the production of IAA. Down-regulation of IAA as signalling was associated with the plant defence mechanisms against a number of phytopathogenic bacteria as evidenced in enhanced susceptibility of plants to the bacterial pathogen by exogenous application of IAA or IAA produced by the pathogen (Spaepen and Vanderleyden, 2011) [55]. IAA was implicated in virtually every aspect of plant growth and development, as well as defence responses. This diversity of function is reflected by the extraordinary complexity of IAA biosynthetic, transport and signaling pathways (Santner *et al*., 2009) [45]. Rhizobacterial IAA was identified as an effector molecule in plant microbe interactions, both in pathogenesis and phyto stimulation (Spaepen and Vanderleyden, 2011) [55].

Table 4: PGPR activities of selected isolates

Sr. No.	Bacterial isolate	IAA (µg/ml)	ACC deaminase (Zone size in mm)	Phosphate (Zone size in mm)	Chitin (Zone size in mm)	Zinc (Zone size in mm)
1	I1 04	40.74	-	-	-	5
2	H5 80	36.15	-	-	-	-
3	H1 23	35.76	-	-	13	14
4	H2 31	35.1	-	-	7	17
5	H1 20	34.78	17	-	10	-
6	H5 81	30.06	-	-	-	-
7	I1 08	28.03	-	-	-	-
8	H4 65	27.77	-	-	10	-
9	I5 59	27.18	-	-	-	-
10	I1 06	26.85	5	-	-	-
11	I2 28	25.28	-	-	7	-
12	H1 24	25.21	-	-	12	-
13	I4 51	25.21	-	-	-	-
14	H4 63	25.15	-	-	-	5
15	H1 03	24.89	-	-	-	-
16	H4 71	24.49	-	-	-	-
17	H1 11	22.6	10	-	-	-
18	I4 58	21.94	-	-	-	-
19	H3 42	21.81	-	-	-	-
20	I5 60	20.83	-	-	-	10
21	I4 57	19.98	-	-	-	-
22	H1 02	19.12	3	7	-	14
23	I5 71	18.8	-	-	-	-
24	H5 86	18.67	-	-	-	-
25	H3 45	17.42	-	-	-	-
26	I4 50	17.36	-	-	-	-
27	H5 79	16.37	-	-	-	-
28	H5 84	16.31	-	-	-	-
29	I4 43	16.24	-	-	-	-
30	H5 92	16.2	-	-	-	-
31	I4 47	15.85	-	-	-	-
32	H1 06	15.52	-	12	-	-
33	H2 32	15.33	-	-	-	-
34	H5 78	14.87	-	-	-	-
35	H3 55	14.74	-	-	-	-
36	I2 22	14.74	-	-	15	-
37	H1 09	14.67	-	-	-	-
38	I4 49	14.54	-	-	-	-
39	H5 77	14.54	5	-	-	10
40	I4 42	14.15	-	-	-	-
41	I2 30	14.02	-	-	-	-
42	I3 37	13.95	-	-	-	-
43	H5 76	13.88	-	12	15	21
44	H5 83	13.56	-	-	-	-
45	H582	13.43	-	-	-	-
46	H5 94	13.23	-	-	-	-
47	H5 75	12.97	-	-	-	-
48	H5 73	12.71	-	-	-	-
49	H1 15	12.25	-	-	-	-
50	H2 33	12.18	7	10	-	20
51	H1 04	11.66	-	-	-	-
52	H3 50	11.66	-	-	-	-
53	I1 15	10.41	-	-	7	-
54	I2 31	10.41	-	-	-	-
55	I4 53	10.35	-	-	-	-
56	H1 12	10.22	-	-	-	-
57	H5 72	10.02	-	15	7	30
58	H4 64	9.56	-	-	-	-
59	H3 56	9.5	-	12	12	-
60	H3 48	9.04	-	-	-	14

(H1= Healthy 1, I1= Infected 1, H2= Healthy 2, I2= Infected 2, H3= Healthy 3, I3= Infected 3, H4= Healthy 4, I4= Infected 4, H5= Healthy 5, I5= Infected 5)

ACC-deaminase activity

The bacterial isolates were screened for ACC deaminase activity (Table 4). Among the 60 isolates, five isolates H1-2, H1-11, H1-20, H2-33 and H5-77 were able to grow on DF (Dworkin and Foster) minimal salt medium supplemented with 3 mm ACC (1-aminocyclopropane-1- carboxylic acid) as a nitrogen source. One H2-33 was able to grown on DF-NH3 medium (Figure 1). Under stress conditions like those generated by salinity, drought, water logging, heavy metals and pathogenicity, the endogenous level of ethylene is significantly increased which negatively affects the overall plant growth. For instance, the high concentration of ethylene induces defoliation and other cellular processes that may lead to reduced crop performance (Saleem *et al*., 2007; Bhattacharyya and Jha, 2012) [44, 4]. Plant growth promoting rhizobacteria which possess the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitate plant growth and development by decreasing ethylene levels, inducing salt tolerance and reducing drought stress in plants (Nadeem *et al*., 2007; Zahir *et al*., 2008) [38, 59]. Currently, bacterial strains exhibiting ACC deaminase activity have been identified in a wide range of genera such as *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobiumetc*. (Shaharoona *et al*., 2007a, b; Nadeem *et al*., 2007; Zahir *et al*., 2008; Kang *et al*., 2010) [46, 47, 38, 59, 25].

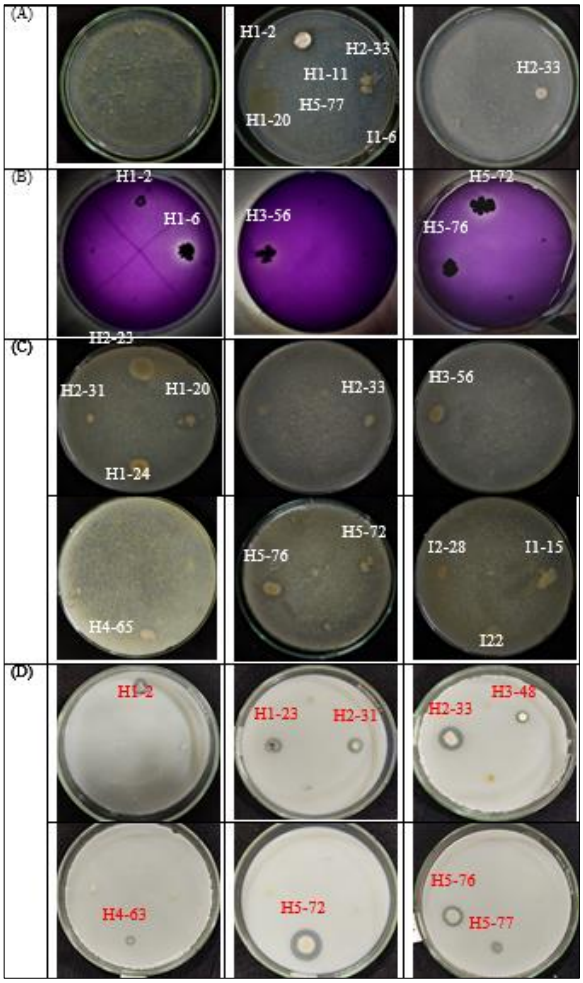

Fig 1: PGPR potentials of selected bacterial isolates (A) ACC deaminase activity on DF-ACC medium and DF-NH_3 medium, (B) Phosphate solubilization (C) chitin hydrolysis (D) Zinc solubilization

Phosphate solubilization ability

The bacterial isolates were screened for phosphate solubilisation (Table 4). Among the 60 isolates, five isolates H1-2, H1-6, H3-56, H5-72 and H5-76 were produced a clear zone around the microbial colonies (Figure 1). Different bacterial genera were reported to have P-solubilizing capacity including strains from bacterial genera, *Acetobacter diazotrophicus* (also known as *Gluconacetobacter diazotropicus*) (Maheshkumar *et al*., 1999) [35] and *Gluconacetobacter* sp. (Chung *et al*., 2005) [11]. The ability to solubilize insoluble inorganic phosphate compounds by *G. diazotrophicus* was studied by Crespo *et al*. (2011) [13]. Use of PSB in agricultural practice would not only off set the high cost of manufacturing phosphate fertilizers, but would also mobilize insoluble in the fertilizers and soils to which they are applied (Chang and Yang, 2009; Banerjee *et al*., 2010) [6, 3].

Chitinase activity

The bacterial isolates were screened for chitin hydrolysis (Table 4). Among 60 isolates, eight isolatesH1-20, H2-23, H2-24, H2-31, H2-33, H3-56, H4-65, H5-72, H5-76, I2-15, I2-22 and I2-28 were screened for chitin hydrolysis (Figure 1). The role of chitinase in the biological control of various fungal pathogens has already been established (Gunaratna and Balasubramanian, 1994; Mathivanan *et al*., 1998; Huang *et al*., 2005; Chang *et al*., 2007) [20, 37, 23, 8]. *Bacillus licheniformis*, *Stenotrophomonas maltophilia*, *Bacillus subtilis* and *Bacillus thuringiensis* were produced good chitinase (Pisano *et al*., 1992. Hoster *et al*., 2005) [41, 22]. Microorganisms were secret a complex of mycolytic enzymes, which was considered biological control agents of plant diseases (Helisto *et al*., 2001, Chang *et al*., 2003) [21, 7].

Zinc solubilization ability

The bacterial isolates were screened for zinc solubilisation (Table 4). Among the 60 isolates, eleven isolates H1-2, H1-23, H2-31, H2-33, H3-48, H4-63, H5-72, H5-76 and H5-77 were produced a clear zone around the microbial colonies (Figure 1). The diameter of these zone was recorded by Kabata-Pendias and Pendias (2001) [24]. Bacteria were improve the plant growth, development by colonizing the rhizosphere and by solubilizing complex zinc compounds into simpler ones, thus making zinc available to the plants. Plants can uptake zinc as divalent cation (Kabata-Pendias and Pendias, 2001) [24] but only a very minor portion of total zinc is present in soil solution as soluble form. Rest of the zinc is in the form of insoluble complexes and minerals (Alloway, 2008) [1]. Prospective zinc solubilizing bacteria for enhanced nutrition and zinc uptake in *Zea mays* L., zinc solubilizing *Bacillus* strains that modulate growth and yield. Zinc biofortification of soybean and wheat were characterized by researchers (Khande *et al*., 2017) [27]. Zinc is important in the oxidative burst defence mechanism of plants (Cakmak, 2000) which reduced disease severity of *F. solani* in wheat, except if zinc was deficient (Khoshgoftarmanesh *et al*., 2010) [28].

Antagonist potential of bacterial isolates against *FoCu*

Isolation of the pathogen (*FoCu*) from infected plant sample

FoCu was isolated from infected plants. Pure culture was obtained on PDA media and identified as *F. oxisporum f*

spcumini by using scanning electron microscopy. *FoCu* was showed pink pigment production and lacto phenol cotton blue mount of pathogen. Length of sickle shape spore was ranged from 10.32 to 23.59 µm, the width was ranged from 3.2 to 3.9 µm. Macroconidia were long, slightly curved or boat shaped, variable in size and smoothly rounded or pointed at the tip, mostly with 2 to 3 septa (Charan *et al*., 2021) [9].

In vitro screening of antifungal activity by dual plate technique

The test for antagonistic activity against the fungal pathogen *FoCu* was conducted using sixty bacterial isolates. Out of the sixty selected isolates, six isolates were found to possess antagonistic activity. Under *in-vitro*H1-2 and H1-23 were showed maximum percent growth of inhibition 39.47% and 52.63% against *FoCu* at 12 days on PDA media (Figure 2). All antagonistic isolates were produced an inhibition percentage range from 31.57% to 52.63% (Figure 3).

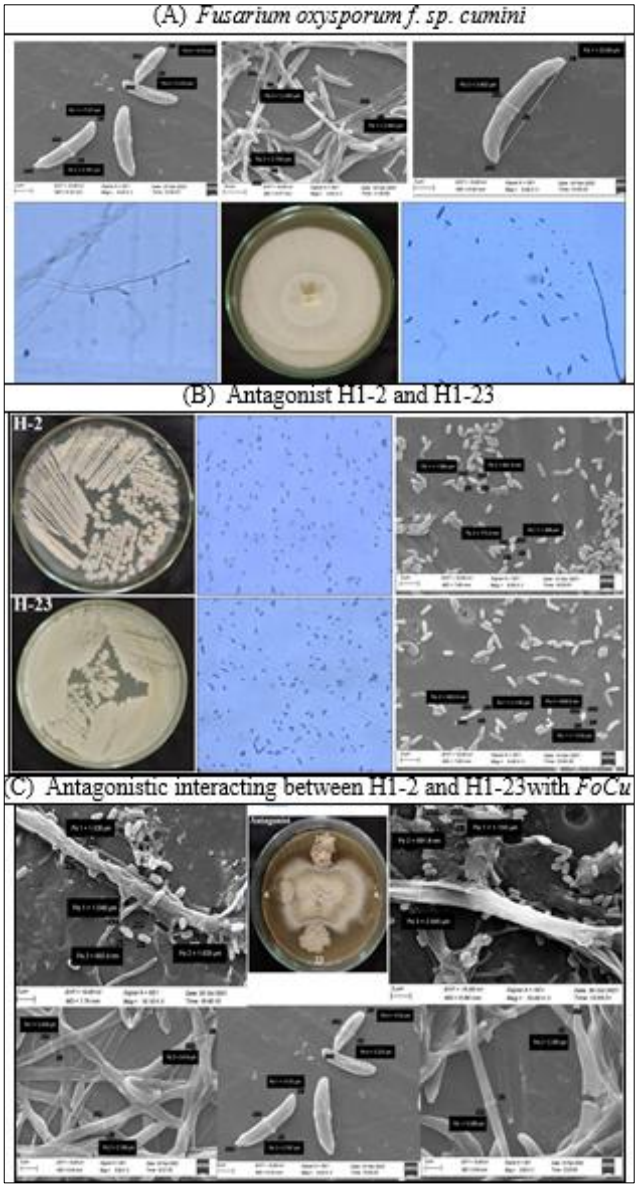

Fig 2: Microscopic characterization of potent antagonist (H1-2 & H1-23) and pathogen *FoCu* (A) Morphological characterization of *Fusarium oxysporum f. sp. Cumini*,(B) Morphological characterization of potent antagonist H1-2 and H1-23 and (C) *In vitro* antagonistic activity of potent bacterial isolates (H1-2 and H1-23) interacting with pathogen *FoCu*

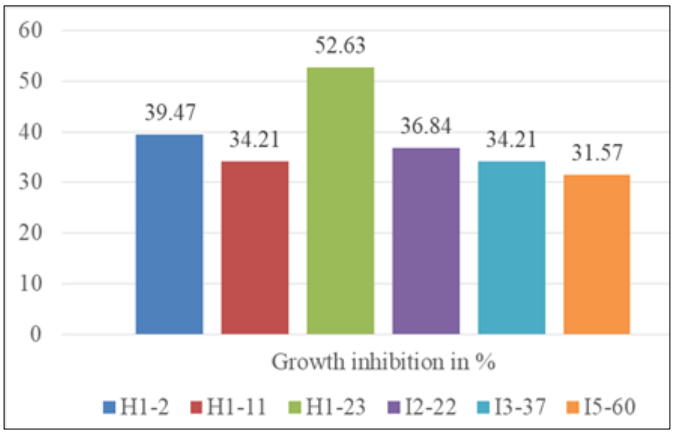

Fig 3: Effect of antagonistic isolates on incidence of fungal wilt *FoCu* under *in vitro* condition

SEM characterization of potent antagonist pathogen and their interaction

We were performed high-resolution SEM image. The micrograph was showed that, potent two strains (H1-2 and H1-23) were rod shape (Figure 2) and length of conidia was 1.594 µm (H1-2) and 2.136 µm (H1-23). Based on SEM observation of spore surface both isolates were considered as *Bacillus subtilus* (Sabio, 2004) [43]. Khan and Gangopadhyay (2012) [26] were reported the antagonistic effect of many bacterial antagonists like *B. subtilis* against different *Fusarium* spp., *viz. F. oxysporum* f. sp. *ciceri, F. oxysporum* f. sp. *udum*, *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *Vasinfectum*. Within *Bacillus* species, *B. subtilis* as the most important species (Raaijmakers *et al*., 2002) [42]. The antagonistic microorganisms such as bacteria are an alternative source for controlling these pathogens. *Bacillus sp*. was considered safe biological agents. Different antagonistic studies with *Bacillus sp*. were done (Utkhede, 1984; Silo-suh, 1994; Kim *et al*., 2003) [56, 52, 29].

The isolates H1-2 and H1-23 were strongly inhibited the growth of *FoCu* under dual culture assay (Figure 2). The *FoCu* mycelia were obtained from the periphery of the inhibition zone of a 12 day old dual culture plate. Observations made under the SEM revealed that the mycelia sample taken from dual culture assay plate was deformed, disintegrating and ruptured. As the antagonist entered the mycelial growth of fungal pathogen, it surrounded the mycelia growth completely and damaged it, reducing apical growth and irregular distortions in the fungal hyphae with reduction in the thickness of mycelia was observed by Shrivastava *et al*. (2017) [51]. Antagonistic isolates Based on mycelia size of *FoCu* fungi was decrease (1.328-2.045 µm) during interaction time to control (2.196 to 3.514 µm). It means new isolated bacteria inhibit a growth of *FoCu* under *in-vitro* condition (Kumar *et al*., 2016) [30].

Efficacy of potent bacterial isolates (H1-2 and H1-23) against *Fusarium* wilt under pot culture

The selected rhizobacterial isolates (H1-2 and H1-23) were tested for their ability to inhibit growth of plant pathogen. Cumin seeds were treated with bacterial isolates in three replications and five treatment like, (i) Cumin seeds soaked in SDW (Control) (T1); (ii) Cumin seeds inoculated with 10^6 cfu/mL of the *FoCu* suspension (T2); (iii) Cumin seeds inoculated with 10^6 cfu/mL of the H1-2 bacterial isolate (T3); (iv) Cumin seeds inoculated with 10^6 cfu/mL of the second

H1-23 bacterial isolate (T4) and (iiv) Cumin seeds inoculated with 10^6 cfu/mL combination of H1-2 and H1-23 isolates (T5).The wilt incidence was considerably suppressed in response to seed treatment with H1-2 and H1-23 (Kumar *et al*., 2016) [30].

Pathogenicity of the isolated cultures of *FoCu* (10^6 cfu/mL) was tested by growing cumin seeds in pot containing pathogen infested soil. Sowing of cumin seeds were completed on 07.12.2021 with three replications and five treatment (Figure 4). Plants were watered on alternate days. The seed emergence was recorded after 17 days of sowing. Observations on number of healthy and wilted plants were recorded at 90 days after sowing (Kumar *et al*., 2016) [30].

f. sp. *cumini* was tested by growing cumin plants in pots containing pathogen infested soil.

Wilt incidence was calculated on the basis of total number of wilted plants and total number of healthy plants. These two antagonists were provided disease control 96.55% in pot trial Chawla and Gangopadhyay (2009) [10]. The disease incidence recorded in these three treatments was statistically at par. The wilt incidence was ranged from 3.45% - 88.80% in response to these two antagonist treatments (Table 5 Figure 4). However, the fungus infested and combination of all two dominant species was effective in controlling the disease incidence (Kumar *et al*., 2016) [30]. Dukare and Paul (2021) [15] were observed that *Pseudomonas* sp. NS 1 and *Bacillus* sp. NS 22 displayed the potential as bio fungicide under *in vitro* condition which is similar to current finding.

Table 5: Antagonist potentials to restrain *Fusarium* wilt infection in cumin GC-4 under pot culture

Treatment	Number of seeds sowing	No. Seeds germination (17days)	Germination in %	No. Healthy plants (90 days)	No. Diseased plants (90 days)	Disease infection %
T_1- Cumin seeds grown under normal soil	30	24.67	82.22	20.00	6.00	18.83
T_2 – Cumin seeds grown under *FoCu* (1 x 10^6 cfu) infected soil	30	21.00	70.00	6.00	20.00	88.80
T_3 – Cumin seeds shocked with bacterial strain H1-2 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil	30	25.67	85.56	22.00	4.00	14.31
T_4 - Cumin seeds shocked with bacterial strain H1-23 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil	30	28.33	94.44	25.00	2.00	5.91
T_5 – Cumin seed shocked with mix bacterial strain H1-2 and H1-23 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil	30	29.00	96.67	27.00	1.00	3.45
S.Em.±		0.33	0.52	0.33	1.19	1.11
C.D. at 5%		1.05	1.63	1.05	3.75	3.50
C.V. %		2.09	5.50	7.53	7.49	2.09

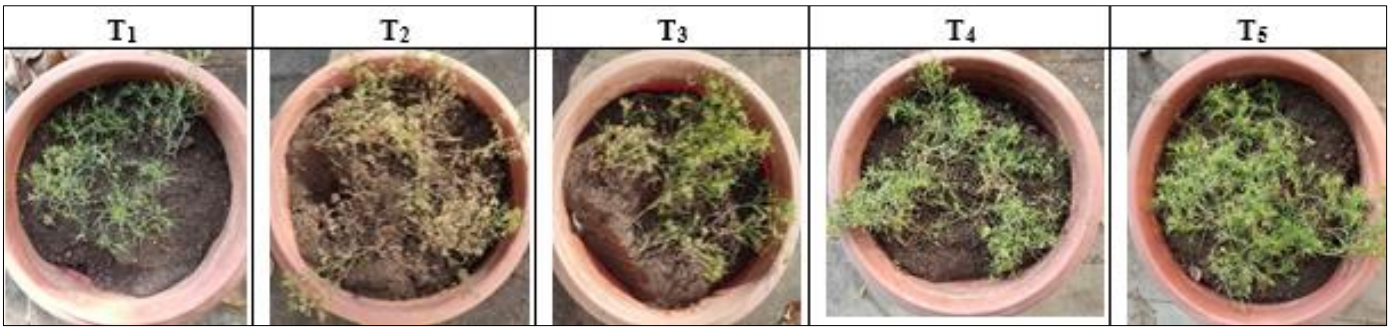

Fig 4: Efficacy of potent bacterial isolates (H2 & H23) to restrain *Fusarium* wilt in pot study [T_1- Cumin seeds grown under normal soil, T_2 – Cumin seeds grown under *FoCu* (1 x 10^6cfu) infected soil, T_3 – Cumin seed shocked with bacterial strain H2 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil, T_4 - Cumin seed shocked with bacterial strain H23 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil, T_5 – Cumin seed shocked with mix bacterial strain H1-2 and H1-23 (1 x 10^6 cfu) for 12hr and sown under *FoCu* infected soil.]

Conclusion

In conclusion, Total 164 isolates were obtained from 10 rhizosphere compering with healthy and infected with *Fusarium oxysporum* f. sp. *cumini* (*FoCu*). After haemolysis test, 60 isolates were found non-pathogenic and further characterised for PGPR and antagonist activity against *FoCu*. 45 isolates were screened for IAA production, 5 isolates were able to grow on ACC deaminase activity, five isolates were able solubilise phosphate, eight isolates were able to hydrolysis of chitin and eleven isolates were solubilize zinc. H5-72 and H5-76 were found potent and common in all three PGPR actives like IAA, Phosphate solubilisation and zinc solubilisation. From 60 bacterial isolates, two bacterial isolates H2 (39.47%) and H23 (52.63%) were suppressed the growth of *FoCu* under *in-vitro* condition. Based on SEM observation both isolates were considered as *Bacillus subtilus*. Potent *Bacillus* isolates derived from microbiome characterisation may be useful as PGPR and antagonist as dual purpose for betterment of cumin cultivation.

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