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Exploring secondary metabolites mediated antagonistic activity of *Bacillus pumilus JRC* for *Fusarium wilt* disease management in Chick pea

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ABSTRACT
The weakening effort in the identification of proficient antifungals exclusively from a original source and increase in microorganisms resistance towards antibiotics has formed an anxious state to control fungal infection. Ubiquitous microbes produce secondary metabolites (SMs) that pays a pivotal in the plant disease management as they possess extensive antagonistic efficacy. Looking towards the significance of the subject, present study was designed to explore extraction, characterization and identification of bacterial secondary metabolites (B-SMs) produced by bacteria isolated from rhizoplane, as it serves ironic source of molecules of biotic interest. In this study total three bacterial strains (JRC, JRC1311 and JRC3007) were isolated and the most potential one was identified as Bacillus pumilis JRC. This bacterial strain was further subjugated to obtain the fractions of SMs F1, F2, F3 (from JRC), using thin layer chromatography (TLC) and UV vis spectrophotometry.. These elements of SMs were identified as iturin 7, pumiliacidin 5C and Ericin, through GC-MS and have shown noteworthy antimicrobial activity against wilt disease causing Fusarium oxysporum. Amongst all these isolates, JRC showed maximum antagonistic activity against phytopathogenic fungus. The study provides strong evidence about microbial population of rhizoplane could be an elevating source of B-SMs (having antimicrobial activity) and furthermore oblige for its enormous exploration in search/isolation of valuable products for the treatment of infections (especially MDR case) in the future.
Keywords: Antagonistic activity, Bacterial secondary metabolites (B-SMs), Phytopathogenic fungi, Plant disease management

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INTRODUCTION

Bacterial secondary metabolites are low molecular mass products with unusual structures. The structurally diverse metabolites show a variety of biocontrol activities like antimicrobial agents, inhibitors of enzymes and antitumor, immune-suppressive and anti- parasitic agents plant growth stimulators, herbicides, insecticides, anthelmintic, etc. [1]–[3]They are produced during the late growth phase (stationary phase/ idiophase) of the bacteria (Ruparelia et al., 2022). The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in logarithmic phase and depressed in stationary growth phases. The bacterial secondary metabolites have distinctive molecular skeleton which is not found in the chemical libraries and about 40% of the microbial metabolites cannot be chemically synthesized [5], [6]. There are five main classes of secondary metabolites such as terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, non-ribosomal polypeptides, and enzyme cofactors [4]. B-SM production by a specific bacterium is indispensable as it works as an inhibitor for other microorganisms (discovery & 2013, 2013; Kelsic et al., 2015) also also B-SMs works as a regulator of the cell differentiation progression and several biosynthetic pathways. Whereas antibiotics are one of the most vital known secondary metabolites discovered from the microbes [9][10]. The current study mainly focused on the isolation and the identification of bacteria from the rhizoplane and the extraction and /or characterization of secondary metabolites from them, which is capable of inhibiting phytopathogenic fungus *Fusarium oxysporum* which causes wilt disease in plant roots.

Thus, the present study fruitfully explored the use of rhizoplane beneficial bio- active products (B-SMs) for disease management in agriculture.

MATERIALS AND METHODS

Study area

The study area was located at jagudan spice research Centre having geographical location 23◦268.5"N80◦23'59.7"E, Gujarat, India.

Primary screening of antagonistic bacteria

Rhizoplane sample was collected as mentioned in the study area from the central Gujarat region. Primary screening was carried out by crowded plate technique by taking 1g of soil in 10 ml of sterile distilled water and soil is then allowed to settle for 10 minutes. 0.1 ml of the aliquot was taken and spreaded on nutrient agar medium containing 1 M NaOH, NaCl 5.0g/L, peptone 10g/L, agar 15g/L, yeast extract 10g/L, pH 7. The plates were incubated at 30±4 °C for up to 24-48 h. After 42 h of incubation, developed colonies were screened for further characterization, while, the petite colonies were screened-out.

Secondary screening of antagonistic isolates

To determine efficacy of screened-in bacteria secondary screening was carried out by dual culture assay against phytopathogenic fungi *Fusarium oxysporum.* Dual culture technique was performed by taking an aliquot of 24-hour screened-in bacterial culture (5 µL), which was transferred on the surface of N.Agar + PDA medium comprising 0.5 M NaOH, NaCl 2.5 g/L, peptone 05g/L, potatoes infused form 100 g/L, Dextrose 10 g/L , yeast extract 05g/L and agar 15g/L, pH 6.0 and incubated at 28°C for 36 hours. Followed to that, a segment of the fungal mycelium (7–10 mm in diameter) was placed in the center of a Petri plate. The fungal mycelium was preliminarily grown in N.Agar + PDA medium medium at 25°C for 7 days and the inhibition size was measured in mm. The isolates possessing antagonistic activity against *Fusarium oxysporum* were further carried out for the production of B-SMs.

Production of B-SMs

Bioactive composites were recovered from the cell free supernant by liquid-liquid extraction with ethyl acetate and water in the ratio 1:1 (v/v). mixture was carried out in a separating funnel by inverting the funnel numerous times, followed by allowing two layers to settle down in separated form. B-SMs dissolve in the solvent (ethyl acetate) layer and separates from the aqueous layer. The aqueous layer was then discarded and solvent containing B-SMs were collected. This method was repeated thrice to extract aqueous free solvent. Extraction was carried out by evaporation till dryness in water bath having temperature of 90 -92◦C to make a profuse extraction. The crude bioactive products were dissolved in a minute volume of ethyl acetate and was placed in oven until drying. The raw bioactive composites were then partially purified by TLC method.

B-SMs extraction

Extraction of B-SMs was carried out by three selected isolates (JRC, JRC1311 and JRC3007) using submerged fermentation in a fermentation media. The fermentation medium used was was KH2PO4: 0.5 g/l, K2HPO4: 0.5 g/l, MgSO4: 0.2 g/l, NaCl: 0.01 g/l, MnSO4: 0.01 g/l, FeSO4: 0.01 g/l, CuSO4: 0.01 g/l, CaCl2: 0.015 g/l, L-glutamic acid: 5 g/l and Glucose: 1%. 5% of the Inoculum medium was added to a fermentation medium and incubated on shaker incubator at 37◦C and 120 rpm for 120 hours. Afterwards B-SMs were separated from cells by centrifugation at 6000 rpm for 20 mins. cell free supernant having B-SMs was then carried out for the further purification process.

Purification of B-SMs

Purification of B-SMs was carried out in a glass column 200 × 10 mm; 6101 – Columns plain with sintered disc and glass stopcock) packed with silica gel (Merck 60, 0.063– 0.2 µm, Ø 3cm × 14cm). samples of raw B-SMs were dissolved in the ethyl acetate with respective volume and applied on the surface of packed glass column containing silica gel. The sample were allowed to diffuse in the glass column and elution was carried out using ethyl acetate as solvent system containing 0.2 M buffer of sodium phosphate. Lastly, fractions of elutades were collected in every 1-minute interval. Afterwards purified B-SMs was then used to determine their antagonistic activity against phytopathogenic *Fusarium oxysporum* by agar well diffusion method [11].

B-SMs characterization by GC-MS

B-SMs characterization was done by taking sample containing concentration of 1.0 mg/mL, prepared in 0.5 mL chromatographic (HPLC) grade methanol followed by sonication for 10 minutes ensuring final volume 1.0 mL with the HPLC grade methanol. The standard and sample solutions were filtered through a 0.22 µm PVDF-syringe filter (SLGV033RS, Millex-GV Syringe Filter Unit, 0.22 µm, PVDF, 33 mm, gamma sterilized) Afterwards using fused silica capillary packed column, prepared samples were introduced in GC by on-column injection (30 m 0.32 mm fused silica capillary column coated with DB-5). Temperature was

increased up to 200 °C to remove the bulk of the solvent and premethylated samples were eluted directly in to MS by increasing temperature to 300 °C. samples were analyzed by capillary column coupled directly to the ion source (inference temperature of 275 °C, inlet pressure at 0.08 MPa) and helium (He) as carrier gas. Analysis time taken for each sample was 30 mins. Characterization was carried out by its retention time by associating with standards under similar environment and quantification of the desired sample were measured by integrated peak area.

Evaluation of in vivo antagonistic activity against Fusarium *oxysporum*

The in vivo antifungal activity of the selected isolates, against fusarium wilt (Fusarium oxysporum) of chick pea plants was examined by culturing soil with *F. oxysporum* in a commercial greenhouse (0.7 m × 5 m each, 15 plants/ plot × three plots). Operative fungicides against *F. oxysporum* was not used through the investigational periods, and each trial was carried out under natural inoculum pressure. The experimentations used a random whole slab strategy with three rows, covering three treatments per row. Every conduct plot was 5 m long, nevertheless the data was composed in the middle of 4 m, leaving 0.5 m on any side as an intermission amongst treated plot. For the fusarium wilt bioassay, plants were inoculated by leaf spray and soil soak using the culture broth of desired isolates that was diluted 200–500-fold from culture broth (1×10^9 CFU/mL) cultivated at 35°C for 24 h in a 5,000-liter fermenter (working volume 3,500 liter, 0.5 vvm, 50 rpm, pH 7.0 ± 0.5). leaf spraying was carried out three times with a handheld sprayer till surfeit. Chick pea disease severity was surveyed at every 5-day breaks after spraying just preceding to yield and the percentage (%) of disease rate was assessed by the mean of triplicate self-regulating experiments. The antagonistic activity of the desired isolates was also surveyed at 5-day breaks just before harvesting in three individual experimentations. The average value of three estimates for both the treatments was transformed in to % as described in (Guo et al., 2014).% control = 100 × [(A—B)/A], where A = area of infection (%) on leaves or sheaths sprayed with Tween 20 solution alone and B = area of infection (%) on treated leaves or sheaths. The antifungal activities of the mutant strain against phytopathogenic fungi were compared with those of the positive control. Disease-control values were expressed as percentage control (± standard deviation) compared to the control [10].

Characterization and identification of rhizoplane bacterial strain

Identification and characterization of the potential three bacterial isolates (JRC, JRC1311, JRC3007) were carried out by morphological, cultural and biochemical tests as described in the [14]. The most potential isolate JRC was carried forward for molecular identification by 16s rRNA sequencing.DNA was isolated from the culture as per the manufacturer's protocol. Quality of DNA was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 357F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer.

RESULTS AND DISCUSSION

Study area

Rhizoplane samples were collected aseptically in sterile plastic bag and kept at 4 ^{0}C until its further used. Sampling site for the bacterial isolation is given in the **fig. 1.**

Screening of bacterial isolates

Total of three isolates JRC, JRC 1311 and JRC3007 were found effective antagonist against fungal phytopathogen *Fusarium oxysporum* after primary and secondary screening by crowded plate and dual culture assay respectfully the zone of inhibition by isolated bacteria is described in **fig: 2.** The antifungal activity of three isolated bacteria from rhizoplane fascinatingly displayed higher inhibition ability than the positive control. The experiential growth pattern of JRC and JRC 1311 isolates exhibited extreme antagonistic activity against the *Fusarium oxysporum*. while jrc3007 showed antagonistic activity comparatively lesser than that of JRC and JRC1311.

Evaluation of in vivo antagonistic activity against Fusarium *oxysporum*

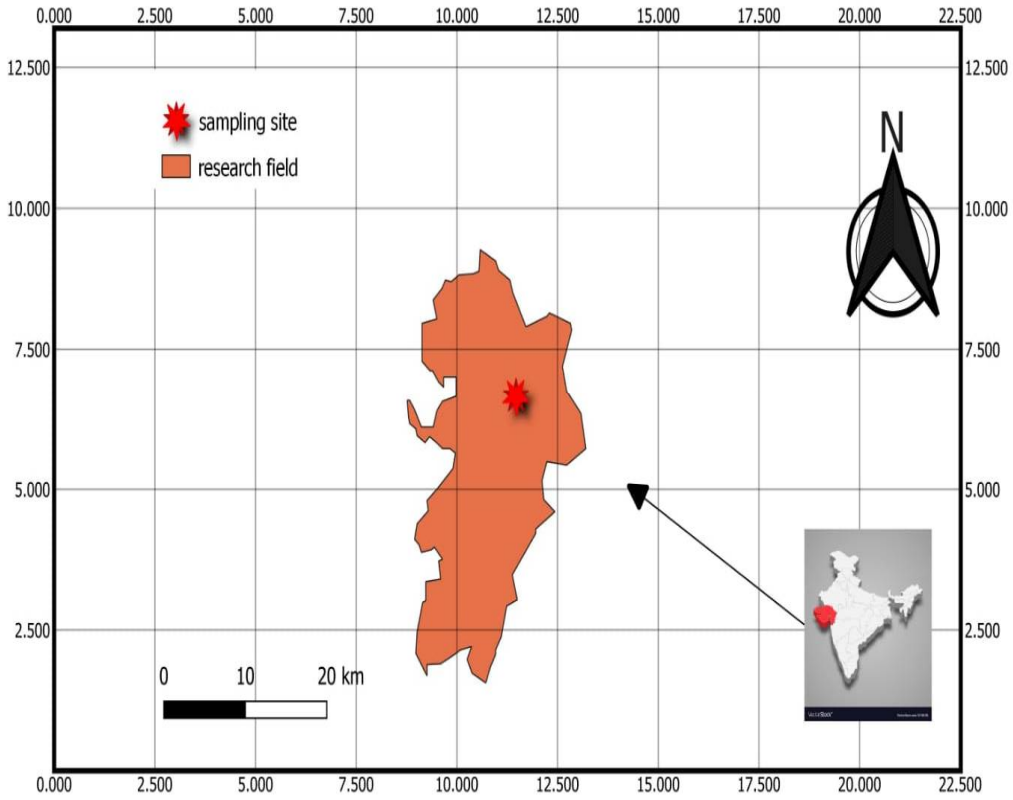
In vivo defensive actions against *fusarium wilt* of chick pea were assessed under commercial greenhouse environments. The disease management efficacy of the JRC bacterial isolate against fusarium wilt was 76.4% at 1.5×10^6 CFU/mL, respectively, whereas the JRC 1311 showed 68.8% at the same concentration and JRC3007 showed 51.7% at same concentration (**Table :1**). The diseases-control activity of the JRC was more stronger than that of JRC1311 , JRC3007 and salable product Cillus available in Korea. None of the toxic effect found during the research periods was detected **(table :1).**

B-SMs extraction and characterization

Most potential isolate on the basis of antagonistic activity, JRC was selected for further characterization for its secondary metabolite production. After submerged fermentation of B-SMs, the extract elution has led to the assortment of some fractions from JRC. These fractions were seen by TLC and UV–vis Spectrophotometer. Major peaks found in the JRC 1 were F1, F2 and F3 from a range of 400 nm–700 nm in reference to elution buffer as blank[14]. Afterwards these fractions were prepared for the GC-MS as discussed earlier. Bioactive fractions peaks were shown in **fig. 4.** Peaks at 2.158 refers to as iturin 7, peaks at 3.235 refers to as pumilicidin 5C as given in the [15], whereas peak at 7.821 refers to as ericin. The results of these peaks were similar to iturin a, a semisynthetic derivative of iturin produced by bacillus subtilis [13]. The peak observed at 3.218 min was found similar to pumilin and the peak at 7.789 min was similar to ericin **(Fig. 4).**

Characterization and identification of rhizoplane bacterial isolates

The most efficient antagonistic bacteria JRC, JRC1311 and JRC3007 were partially identified on the basis of



morphological and cultural characterization by compound light microscope under oil immersion lens 100x and by

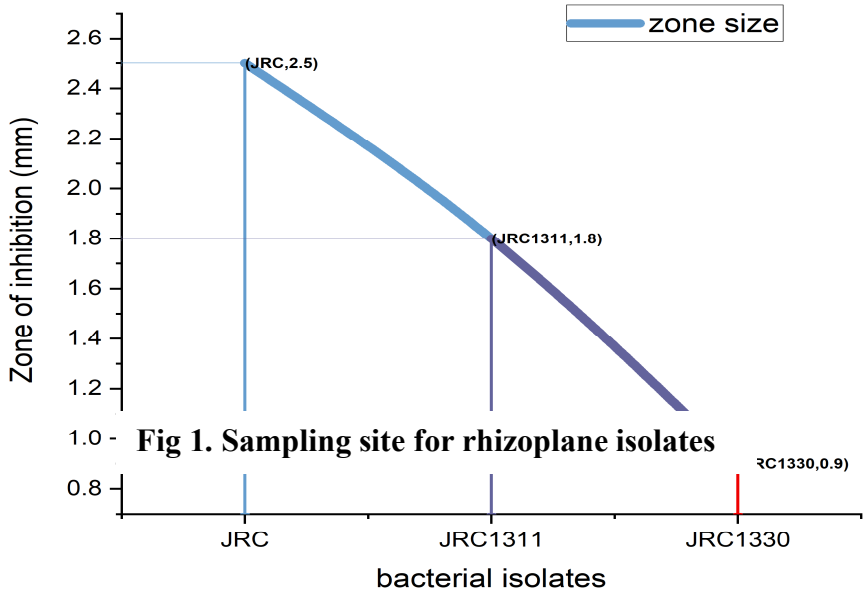


Fig 1. Sampling site for rhizoplane isolates

streaking all the three isolates on N. agar plate at 37 ^{0}C for 24 hours to characterize it respectively as given in the **Table :3.** Isolates were also characterized on the basis of their metabolic activities i.e. assimilation, utilization, salt tolerance and pH tolerance as given in the **Table : 2.**

16S rRNA gene amplification, analysis and phylogeny

The gene specific primers F255 was used for the amplification of 16S rRNA gene for the desired isolate JRC and were resolved on agrose gel electrophoresis. These goods of PCR were then subject to 16S rRNA sequences (bacteria) BLASTN (megablast algorithm). the lengths of PCR products were found to be 1500 bp. On the basis of taxonomic and phylogenic characterization, JRC was found to be 99% similar to *Bacillus pumilus.* Especially, the E-value for the BLAST results was found to be nil, demonstrating likeness in sequences with that database. Phylogenic characterization was intended for determination of possible molecular affiliations of the isolates by classifying the neighboring relative. Rhizoplane isolate JRC **(fig. 5)** was identified as *Bacillus pumilus JRC* a member of bacillus genus belongs to gram positive family that can grow at eclectic mesophilic temperature range (20–45 °C), which is reliable with the current study (Bryant et al., 1958).

Fig 2. Antagonistic activity of bacteria against *Fusarium oxysporum* (zone size in mm)

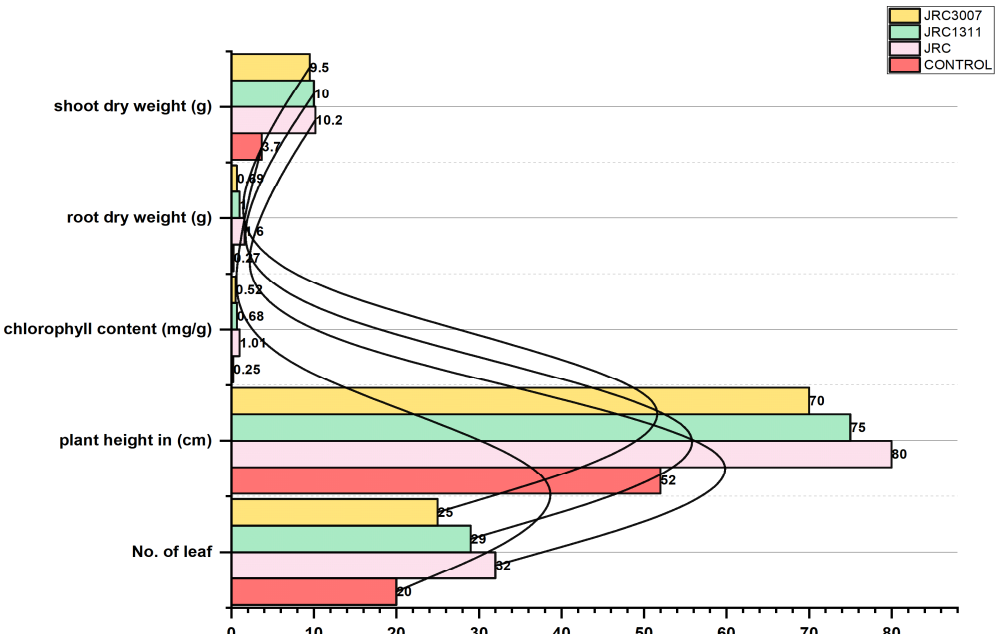


Fig. 3. In-vivo pot experiments for disease management study by bacterial isolates in chick pea.

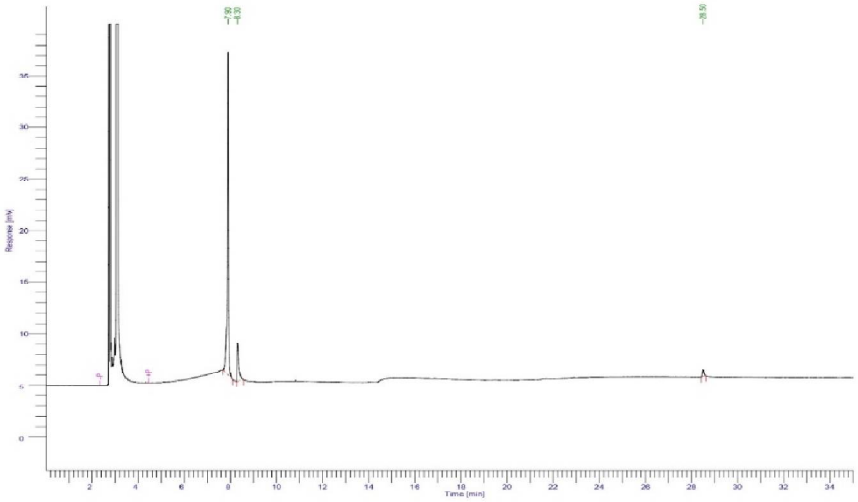


Fig. 4. GC-MS profiling of ethyl acetate extracts of B-SMs peaks F1, F2 and F3

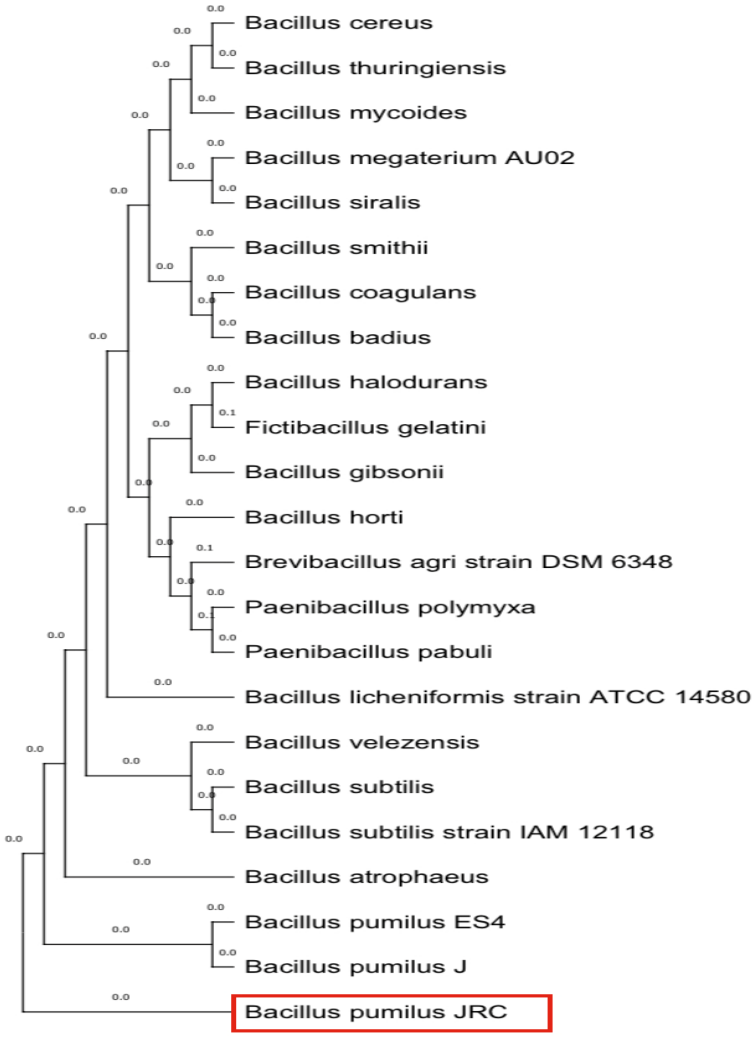


Fig. 5. Molecular characterization of JRC

Table: 1 In vivo disease suppression of *B. pumilus* JRC strain against *fusarium wilt* of chick pea.

Cultural characteristics	JRC	JRC1311	JRC3007
Size	medium	medium	small
Shape	circular	irregular	circular
surface	smooth	rough	Smooth
Opacity	opaque	opaque	translucent
edge	entire	uneven	Entire
Pigment	nil	Light pink	Nil
Elevation	Raised	flat	raised
consistency	mucoid	mucoid	mucoid
Microscopic characteristics			
Size	big	big	Small
shape	rod	rod	rod
Arrangement	In the chains	In the chains	scattered
Gram reaction	+ve	+ve	-ve

Table: 2. Biochemical profiling of rhizoplane isolates

Treatment #1		Control value (%) #2 *Fusarium oxysporum*
JRC	1000 *	60.4± 1.50 #2
	500*	76.4± 0.92#1
JRC1311	1000 *	58.4± 1.50 #2
	500*	68.8± 0.92#1
JRC3007	1000 *	59.4± 1.50 #2
	500*	51.1± 0.92#1
cillus	500*	50.1± 0.92#1
Control	-	**-**
Note: 1000× and 500×: 1,000-fold dilution (1 × 10^6 CFU/mL) and 500-fold dilution (2 × 10^6 CFU/mL) of each culture broth (1 × 109 CFU/mL) produced from JRC, JRC1311 & JRC3007 respectively. #2 The values were expressed as the mean ± SD for three independent experiments (P = 0.05).		

Table :3. Cultural and microscopic examination of of rhizoplane isolates

assimilation	JRC	JRC1311	JRC3007
Glucose	a	a	a
Lactose	c	b	c
Xylose	b	b	c
Mannitol	b	b	b
sucrose	a	a	b
utilization			
urea	b	b	b
Citrate	b	b	b
Arginine	a	a	b
Lysine	a	a	b
Glycine	a	a	a
Ammonium chloride	a	a	b
Salt tolerance			
1%	a	a	a
3%	b	b	b
5%	b	c	c
7%	c	-	c
pH tolerance			
5	b	b	b
7	a	a	a
9	c	c	c
11	-	c	-
Note: (a)= high growth, (b)= moderate growth , (c)= poor growth and (-) = no growth.			

CONCLUSION

As a result of extensive rise in the case of multidrug resistance phenomenon, it is envisioned that investigation towards affective bioactive antibiotics and other B-SMs are straightaway requirement that might be convenient in the field for aim based dealing approaches that is discovered from the ubiquitous microbes.in this study , mainly three bacteria (JRC, JRC1311 and JRC3007) possessing antagonistic activity against phytopathogenic fungus were screened from the rhizoplane and their B-SMs were characterized by GC-MS. The B-SMs obtained from these bacterial isolates were iturin 7, pumiliacidin 5C and Ericin. These B-SMs could form an admirable approach also for the medical microbiology for the development of effectual and harmless control for multi drug resistant (MDR) pathogens-SMs could also form an alternative for the numerous disease control in the agriculture field.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

AVAILABILITY OF DATA AND MATERIALS

The manuscript comprises all the applicable data in the text, tables and in the figures.

CONSENT FOR PUBLICATION

JR and CKJ initiated the idea of research. JR and AR wrote the manuscript. JR, AR and CKJ reviewed and finalized the whole manuscript.

CONFLICTS OF INTERESTS

All authors declare that there is no any conflict of interests for this publication.

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