Biological Forum
an International Journal 17(7): 116-127(2025)
ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Exploring Higher Root Rhizospheric Soil as a Source of Potential Bacterial Antagonist Against Root Rot Fungi *Macrophomina phaseolina* Infecting Black Gram

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(Received: 20 April 2025; Revised: 29 May 2025; Accepted: 25 June 2025; Published online: 11 July 2025)
(Published by Research Trend)

ABSTRACT: Soil-borne pathogens like *Macrophomina phaseolina* pose a significant threat to black gram (*Vigna mungo*), a vital pulse crop in India. In this study, 21 rhizospheric bacterial isolates were initially screened, and 12 were characterized morphologically. Using the dual culture method, five isolates AC 2, AN 3, AF 4, JB 8, and NP 9 demonstrated strong antagonistic activity, with growth inhibition exceeding 50%, against *M. phaseolina*. These isolates underwent detailed morphological, biochemical, and molecular analyses, including 16S rDNA sequencing and ARDRA profiling. The isolates were identified as *Bacillus licheniformis* (AAU BCM 1), *Bacillus stratosphericus* (AAU BCM 2), *Pseudomonas aeruginosa* (AAU BCM 3), *Pseudomonas azotoformans* (AAU BCM 4), and *Stenotrophomonas* sp. (AAU BCM 5). Several of these antagonists produced key antifungal enzymes (lipase, protease, chitinase) and hydrocyanic acid (HCN), and some exhibited ACC deaminase activity, enhancing their potential as plant growth-promoting rhizobacteria. Scanning electron microscopy confirmed the antagonistic action of *B. licheniformis* against *M. phaseolina* hyphae. Antibiotic resistance profiling indicated strong survivability of the isolates in soil environments. These findings highlight the promise of native rhizospheric bacteria as sustainable biocontrol agents against *M. phaseolina* in black gram cultivation.

Keywords: Black gram, *Macrophomina phaseolina*, antagonistic rhizobacteria, ARDRA, 16S rDNA sequencing, Scanning electron microscopy, Dual culture assay.

INTRODUCTION

Black gram (*Vigna mungo* L. Hepper) is popularly known as urd bean, black maple, mash is an annual, semi erect to spreading herb belonging to the Leguminosae family. It is grown in tropical and sub-tropical countries as *Kharif* crop (Gopalan *et al*., 1971). Among the pulses, black gram is fourth most important crop in terms of area and the third in terms of production in India. There are number of fungal, bacterial and viral diseases which affect the black gram. Among them the root rot disease caused by *Macrophomina phaseolina* is becoming more serious because of its seed and soil borne nature (Indira and Gayatri 2003). *M. phaseolina* is a soil-dwelling fungus present all over the world affecting 500 plant species belongs to more than 100 families. It causes various diseases such as stem and root rot, charcoal rot and seedling blight (Marquez *et al*., 2021). *M. phaseolina* is identified by hyaline hyphae with septa and the main hyphae often producing branches at a right angle, with constriction at the point of origin. The fungus exhibits two distinct: a sclerotial phase (*Rhizoctonia bataticola*) that forms microsclerotia and a pycnidial phase (*M. phaseolina*) that develops pycnidia in host tissues (Nicholson *et al*., 1972). Microsclerotia are spherical, oval or oblong masses of hardened fungal mycelium which are light brown when young and become darken (brown to black) with ageing (Lakhran *et al*., 2018). The pathogen targets the basal area of the plant (Arora *et al*., 2012). Its microsclerotia, which are produced in senescing shoot tissues, can last in soil for a very long time (Mayek-Perez *et al*., 2002). Disease management is challenging because the fungus is soil-borne. The fact that sclerotia are soil-borne and have complicated survival mechanisms can make chemical control inconsistent, challenging and uneconomical (Gul *et al*., 1989). Additionally, having several disadvantages like pesticide residues in crop produce and soil, pollution of

ground water, emergence of resistant races and killing of non-target beneficial microbes, *etc*.

As a result, biocontrol agents provide an environment-friendly alternative to chemicals in the management of soil-borne (Ramesh and Korikanthimath 2006). Among the wide range of beneficial Biocontrol of soilborne phytopathogens by antagonistic rhizobacteria offers a nonpolluting complement and an alternative to existing disease management strategies (Senthilkumar *et al*., 2009).

The rhizosphere is the area of the soil where a plant's feeder root can be found, it has a zone of intense microbial activity that creates a nutrient pool where vital macro and micronutrients can be found (Subba Rao and Dommergues 1998). Bacterial antagonist has beneficial effect on plant development as well as have beneficial effect on suppressing phytopathogenic microorganisms (Kloepper *et al*., 1989; Son *et al*., 2014). The direct growth-promoting mechanisms include nitrogen fixation, phosphorus solubilization, sequestering of iron through synthesis of siderophores, production of phytohormones like auxins [indole acetic acid (IAA)], cytokinins, gibberellins and lowering the concentration ethylene (Kloepper *et al*., 1989; Glick *et al*., 1999). The indirect method includes the production of antibiotic, depletion of iron from the rhizosphere, antifungal metabolites synthesis, production of fungal cell wall degrading enzymes, competition for sites on roots and the induction of systemic resistance. They synthesize the enzyme 1-aminocyclopropane1-carboxylate (ACC) deaminase that hydrolyze ACC which is an ethylene precursor, which leads to early senescence. It promotes seedling germination and growth by lowering the ethylene concentration (Glick *et al*., 1999). A volatile compound called hydrogen cyanide (HCN), which is produced by bacterial antagonists, which have biocontrol activity against plant pathogens (Siddiqui *et al*., 2007). HCN protects the plants from soil-borne diseases by impeding cytochrome oxidase and other metallo-enzymes of the pathogens (Voisard *et al*., 1989; Blumer and Haas 2000). Looking in to requirement of time to encourage the use of antagonistic, plant growth-promoting and defense-inducing rhizobacteria in plant disease management, this work has been carried out.

MATERIAL AND METHODS

A. Soil sampling

Rhizospheric soil from roots of different trees like neem, ber, palm, gum, acacia, aonla, mango, coconut, false ashoka, palash, jamun and banyan tree were collected from North, South, Central and Saurashtra region of Gujarat during Summer-*Kharif*, 2022. Four rhizospheric soil samples were taken per tree, approximately 50 cm away from the trunk and at a depth of 5-10 cm, where most of the feeder roots could be found. Samples from a same tree were mixed with the help of hand shovel to obtain one composite sample of rhizosphere soil per tree. After each sampling, samples were brought to the laboratory and immediately processed upon arrival. Loose soil was removed from the roots by shaking them gently and the remaining soil, adhere strongly to the roots, was considered as rhizospheric soil (Guevara-Avendano *et al*., 2018). The soil was kept into a plastic bag and labelled with the name of tree, location, collection date and name of sampler.

B. Isolation, purification and cultural characterization of rhizobacteria

Serial dilution technique was followed to isolate the rhizobacteria where, 10 g of soil was suspended in 90 ml of sterile distilled water in 250 ml conical flask. The soil suspension was shaken in an orbital shaker cum incubator at 28 ±2 °C and 120 rpm for 25-30 min. The suspension was then allowed to stands till setting of soil particles for 30 min. From upper liquid portion, dilution upto 10-8 were prepared. From 10-4 to 10-8 dilutions, 1 ml of suspension was serially diluted and 100 µl of that suspension was spreaded on to the sterile Nutrient Agar (NA) medium and King's B (KB) medium. Plates were incubated at 28±2°C for 48 h in a BOD incubator. After the 48 hrs. plates were observed for number of rhizobacterial colonies of different morphological forms (Dashti *et al*., 2021). Rhizobacterial isolates having different morphological appearance on agar plates were selected and purified by the four-flame streaking method on NA/KB medium. The isolates were stored in refrigerator at 4°C for further study. For cultural characterization, colony characters like shape, margin, elevation, texture, opacity and pigment were recorded from inoculated plates.

C. Isolation and purification of pathogen

The diseased stem/roots/bark of black gram showing typical symptoms bearing microsclerotia of the fungus were collected from Agronomy farm, Anand Agricultural University, Anand and the fungus was isolated using standard tissue isolation procedure given by Tuite (1969). The pure culture, thus obtained by subsequent single hyphal tip method and kept at 28±1 °C for further investigation. Subsequent, sub-culturing was done once in 20 days on PDA slants and preserved in a refrigerator at 4°C for further experimental use.

D. Dual culture method

The antagonistic activity of the bacterial isolates against *M. phaseolina* was evaluated by dual culture method (Foldes *et al*., 2000) with some modifications. With the help of sterile cork borer, a disc agar having fungal growth of pathogen from plate was taken and placed at the centre of the fresh NA-PDA (20 g dextrose, 20 g agar powder, 5 g peptone, 3 g beef extract, 5 g NaCl) plate. Test bacterial culture suspension (50 µl) was inoculated in the four wells 3 cm away from fungal disc and kept for incubation at 30±2°C for seven days. The Petri plates with the only pathogen at the centre served as control (Pathogen check). Inhibition of fungal growth was recorded at 5th and 7th days after co-incubation and compared with pathogen check. The radial growth of mycelium was measured and per cent inhibition were calculated by using the formula given by Vincent (1947).

$$\text{Per cent inhibition (PI)} = \frac{C - T}{C} \times 100$$

Where, C is the radial growth of fungus in control (pathogen check) plate (mm), T is the radial growth of fungus in treatment plate (mm). Rhizobacterial isolates showed more than 50 per cent growth inhibition were selected for further morphological, biochemical and molecular characterization (Foldes *et al*., 2000).

E. Morphological characterization

Efficient antagonistic bacterial isolates were tested for fluorescent test and Gram's reaction described by Aneja (2007). Observation of yellowish green fluorescent pigment produced by bacterial isolates was recorded under UV light (365 nm) indicates positive result. While positive and negative results of Gram's staining were considered based on colour changes as purple and pink, respectively.

F. Biochemical Characterization

HCN Production: Efficient antagonistic bacterial isolates were screened for HCN production by the methodology described by Castric (2009). The isolates were inoculated on the nutrient media plates containing 4.4 g glycine per liter. To the top of the plate, Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5 per cent picric acid solution was placed and sealed with parafilm. The plates were incubated at 30°C for 4 days and observed for a colour change of the filter paper from deep yellow to reddish-brown which indicates production of HCN.

ACC deaminase production: Qualitative screening of efficient antagonistic bacterial isolates for ACC deaminase enzyme production was carried out based on their ability to use ACC (1- Aminocyclopropane-1-Carboxylate) as a sole nitrogen source in the sugar free minimal salt medium. Cultures were spot inoculated on Petri plates containing DF salt minimal medium (KH_2PO_4 1.36 g, Na_2HPO_4 2.13 g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2.2H_2O$ 0.7 g, $FeSO_4.7H_2O$ 0.2 g, $CuSO_4.5H_2O$ 0.04 g, $MnSO_4.H_2O$ 0.02 g, $ZnSO_4.7H_2O$ 0.02 g, H_3BO_3 0.003 g, $CoCl_2.6H_2O$ 0.007 g, $NaMoO_4.2H_2O$ 0.004 g, Glucose 10 g, Agar 15 g, Distilled water 1000 ml) (Dworkin and Foster 1958) supplemented with 3 mM ACC substrate. Plates containing DF minimal medium without ACC was served as negative control and with $(NH_4)_2SO_4$ (2.0 gm/L) as a nitrogen source, served as positive control. The plates were incubated for 3 days at 28±2°C. Growth of strains on ACC supplemented plates were compared with positive and negative control plates. Bacterial isolates growing well on ACC plates were considered as ACC deaminase enzyme producers (Daun *et al*., 2009).

Cell wall degrading enzyme production

Chitinase: Chitinolytic activity was determined by streaking bacterial isolates on Nutrient agar medium supplemented with 0.2% colloidal chitin (Sessitsch *et al*., 2004). Zone of chitin digestion was considered as positive result.

Protease: Protease production was determined using skimmed milk agar (Skim milk powder 10 g, Peptone 5 g, Agar 15 g, Distilled water 1000 ml). Bacterial isolates were spot inoculated and incubated for 2 days at 30±2°C. Proteolytic activity were identified by clear zone around the colony (Smibert *et al*., 1994).

Lipase: Lipolytic activity was determined by streaking bacterial isolates on Tributyrin agar (Peptone 5 g, Beef extract 3 g, Tributyrin 10 ml, Agar 15 g, Distilled water 1000 ml) plates (Lawrence *et al*., 1967).

Antibiotic resistance test: Efficient antagonistic bacterial isolates were grown in Nutrient broth at 30±2°C for 24 h and 0.1 ml of each isolate was spread on nutrient agar plates. The discs of antibiotics were kept on inoculated plates at equidistance from each other. Plates were incubated at 30±2°C for 48 h. Zone of inhibition was observed for each antibiotic disc and diameter from two sides was recorded. Based on diameter of zone of inhibition, the isolates were classified into four types; highly resistant < 5 mm; resistant 5-10 mm; susceptible 10-20 mm and highly susceptible > 20 mm (Abaidoo *et al*., 2002).

G. Molecular Characterization

Genomic DNA isolation: Genomic DNA of the efficient antagonistic bacterial isolates was isolated following the method of George *et al.* (1996). 16S rDNA gene amplification and Amplified Ribosomal DNA Restriction Analysis (ARDRA). The amplification of the 16S rDNA gene by PCR was performed in PCR reaction mixture (25 µl) containing 2.5 µl Taq Buffer (10 X), 0.5 µl dNTPs (2.5 mM each) mix, 2.0 µl Template DNA (25 ng/µl), 0.2 µl Taq polymerase (5U/µl), 17.8 µl Millipore Sterilized Water using the following primer 1.0 µl Primer 1 (27 F-5'-AGA GTT TGA TCC TGG CTC AG-3') and 1.0 µl Primer 2 (1492 R- 5'-GGT TAC CTT GTT ACG ACT T-3') and the primers synthesized at Eurofins Genomics, Bengaluru. These primers designed on the basis of conserved sequences of Weisburg *et al*. (1991), were located at the extreme 50 and 30 of the 16S rDNA gene, respectively, allowing an approximately 1500-bp DNA fragment to be amplified. After mixing of all the components polymerize chain reaction was carried out in Mastercycler Personal (Eppendorf, Germany) with initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min) and extension (72°C for 2 min) and final extension step at 72°C for 10 min. PCR amplified products were run on agarose gel electrophoresis. 16S rDNA gene PCR product (10 µl) of each efficient antagonistic bacterial isolates was used to carry out the restriction digestion with three different tetra-cutter restriction enzymes (*Alu* I, *Rsa* I and *Taq* I). The reaction condition and components are described in Table 1. The products resulting from digestion were observed using a gel documentation system.

Table 1: Reaction component and conditions of ARDRA.

Component/Condition	*Alu I* (5 U/µl)	*Rsa I* (5 U/µl)	*Taq I* (5 U/µl)
1. Reaction Component (µl)			
a. 10X assay buffer	2.00	2.00	2.00
b. Restriction enzyme	1.75	1.75	1.75
c. Nuclease free water	6.25	6.25	6.25
d. PCR product	10.00	10.00	10.00
Total Volume	**20.00**	**20.00**	**20.00**
2. Reaction Conditions:			
***Alu I* :** All the reaction mixtures were incubated at 37°C for 15 min and enzymes were deactivated at 80°C for 20 min after incubation.			
***Rsa I* and *Taq I* :** All the reaction mixtures were incubated at 37°C for 3 hrs and enzymes were deactivated at 80°C for 20 min after incubation.			

Data scoring, data analysis and dendrogram: Relationship between bacterial isolates were established using data from restriction enzyme that adequately differentiated bacterial isolates. The data were scored based on the presence (1) or absence (0) to generate input matrix for analysis. If a product was present in an isolate, it was considered as '1' and if absent, was considered as '0'. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA). The data generated and dendrogram was drawn in NTSYS-pc 1.8 program (Applied Biostatistics Inc., Setauket, NY, USA) (Abaidoo *et al*., 2002).

Bacterial identification and 16S rDNA sequence analysis: Partial 16S rDNA gene sequencing was carried out for efficient antagonistic bacterial isolates and was performed using the ABI 3730 genetic analyzer at Eurofins Genomics, Bengaluru. The 16S rDNA gene sequences were assembled using MEGA 4 software, compared with other strains using NCBI BLAST (National Centre for Biotechnology Information) analysis (Altschul *et al*., 1997) for identification purpose and comparison of homologies of isolated strains with previously characterized strains.

Scanning electron microscopy: Scanning electron microscopy (SEM) of dual culture assay of *Bacillus licheniformis* against *M. phaseolina* was done under in-vitro conditions. Fungal mycelia of *M. phaseolina* growing towards the inhibition zone of *B. licheniformis* under dual culture assay after 4-5 days were cut from the zone and placed on cover glasses. These were exposed to osmium tetraoxide (2%) for 24 hrs at 20°C and transferred to copper stubs over double adhesive tape and scanned in SEM at Department of Biotechnology and Biochemistry, Junagadh Agricultural University, Junagadh, Gujarat.

H. Statistical analysis

The standard methods of analysis of variance for completely randomized design. The appropriate standard error of mean (S. Em. ±) was computed in case of design. For the treatment effect, which were found to be significant, the critical difference (CD) at 5 per cent level of probability was worked out. The test of significance among the treatments were worked out by "F" test.

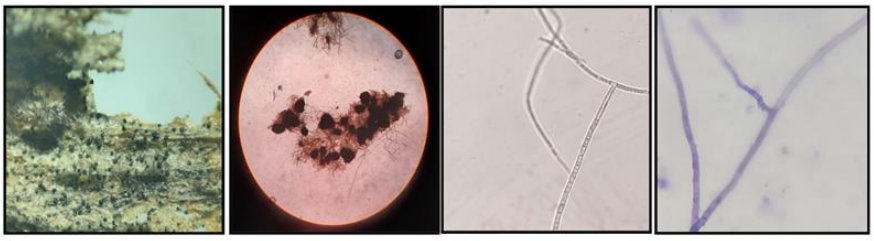


Figure. Cultural and morphological characteristics of *M. phaseolina* **(a)** Sclerotia on bark Stereomicroscopic view; **(b)** Mass of sclerotia**; (c)** Mycelium showing right angle branching).

RESULTS AND DISCUSSION

A. Isolation and Purification of rhizobacteria

Total 21 rhizobacterial isolates were obtained from rhizospheric soil of trees following serial dilution techniques. Pure colony were obtained by four flame streaking method on NA/KB medium. Isolates were designated as NP 1 (Navsari, Palm tree), AC 2 (Anand coconut tree), AN 3 (Anand, Neem tree), AF 4 (Anand, false Ashoka tree), AN 5 (Anand, Neem tree), NP 6 (Navsari, Palm tree), NP 7 (Navsari, Palm tree), JB 8 (Junagadh, Banyan tree), NP 9 (Navsari, Palm tree), BN 10 (Banaskantha, Neem tree), BN 11 10 (Banaskantha, Neem tree) and BA10 (Banaskantha, Acacia tree) 12.

B. Cultural characterization of rhizobacteria

Among 21 rhizobacterial isolates, twelve isolates were selected for their different colonial characterization based on different colony characteristics by growing on NA/King's B medium followed by observation after 48

hrs of inoculation. All the isolates showed variable colonial characteristics as depicted in Table 2. NP 1 showed off-white, AF 4 showed green, NP 6 and BN 12 showed yellow whereas JB 8 and BN 11 showed light-yellow pigmentation on NA/King's B medium. NP 1, AN 3, NP 6, JB 8 and BN 10 formed round to small round shape, entire margin, raised to slightly raised or flat elevation, smooth texture and opaque colony. Whereas remaining isolates 4C 2, AF 4, AN 5, NP 7, NP 9, BN 11 and BA 12 depicted irregular colony shape with wrinkled or undulated margin, flat or raised elevation, rough or smooth texture and translucent or opaque colony.

Table 2: Colonial characterization of bacterial isolates.

Isolates	Colonial characteristics					
	Shape	**Margin**	**Elevation**	**texture**	**Opacity**	**Pigment**
NP 1	Round	Entire	Slightly raised	Smooth	Opaque	Off-white
AC 2	Irregular	Wrinkle	Umbonate	Rough	Opaque	No
AN 3	Round	Entire	Raised	Smooth	Opaque	No
AF 4	Irregular	Entire	Flat	Smooth	Translucent	Green
AN 5	Irregular	Wrinkle	Slightly raised	Rough	Opaque	No
NP 6	Round	Entire	Flat	Smooth & slimy	Translucent	Yellow
NP 7	Irregular	Wrinkle	Umbonate	Rough	Opaque	No
JB 8	Round	Entire	Raised	Smooth	Translucent	Light-yellow
NP 9	Irregular	Undulated	Slightly raised	Rough	Opaque	No
BN 10	Small round	Entire	Raised	Smooth	Opaque	No
BN 11	Irregular	Wrinkle	Slightly raised	Rough	Opaque	Light-yellow
BA 12	Irregular	Undulated	Flat	Smooth	Translucent	Yellow

C. Isolation and purification of pathogen

The fungal colony of pathogen exhibited rapid growth on PDA medium, reaching a diameter of 90 mm within seven days at a temperature of 30±1 °C. The mycelial growth was linear and fluffy, initially appearing as a dirty white colour when young, but later turning to a blackish-grey. The colony also developed black round microsclerotia. The hyphae of the fungus branched at right angles, with a noticeable constriction at the point of origin of the hyphal branches. Additionally, the constriction was positioned close to the branching point. Based on cultural and morphological characters, the pathogen primarily identified as *Macrophomina phaseolina* (Tassi) Goid.

D. Dual culture method

The data in Table 3 shows that all bacterial isolates significantly inhibited the mycelial growth of *M. phaseolina* (35-75%). Significantly minimum mycelial growth of *M. phaseolina i.e*. 11.33 mm was observed in AF 4 with maximum growth inhibition of 74.81 per cent, followed by JB 8 with 11.67 mm of mycelial growth and 74.07 per cent growth inhibition, AC 2 with 12.67 mm of mycelial growth and 71.85 per cent growth inhibition, followed by NP 9 with 13 mm mycelial growth and 71.11 per cent growth inhibition, AN 3 with 13.33 mm mycelial growth and 70.37 per cent growth inhibition. Bacterial isolates (AC 2, AN 3, AF 4, JB 8 and NP 9) showed more than 50 per cent growth inhibition (Fig. 1) were subjected to morphological, biochemical and molecular characterization.

Table 3: Bio efficacy of antagonistic bacterial isolates against *M. phaseolina* under in-vitro conditions.

Tr. No.	Treatments	*M. phaseolina*	
		Radius of mycelium (mm)	**Growth inhibition (%)**
T_1	NP 1	24.33^{de}	45.93
T_2	AC 2	12.67^{g}	71.85
T_3	AN 3	13.33^{g}	70.37
T_4	AF 4	11.33^{g}	74.81
T_5	AN 5	29.00^{b}	35.56
T_6	NP 6	25.00^{cde}	44.44
T_7	NP 7	26.33^{bcd}	41.48
T_8	JB 8	11.67^{g}	74.07
T_9	NP 9	13.00^{g}	71.11
T_{10}	BN 10	27.67^{bc}	38.52
T_{11}	BN 11	26.67^{bcd}	40.74
T_{12}	BA 12	23.00^{ef}	48.89
T_{13}	*M. phaseolina* (Pathogen check)	45.00^{a}	-
S. Em. ±		0.91	-
C. D. at 5%		Sig.	-
C. V. (%)		7.13	-

Note: Treatment means with letter/ letters in common are not significant by Duncan's multiple range test at 5% level of significance

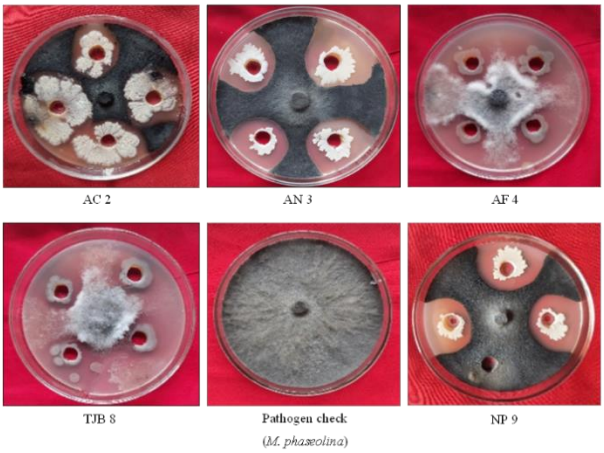


Fig. 1. Bio efficacy of efficient antagonistic bacterial isolates against *M. phaseolina* in-vitro.

E. Morphological characterization

Five efficient antagonistic bacterial isolates were inoculated onto the sterilized NA/King's B medium contained in Petri plates and kept for incubation for two days. Among them AF 4 showed dark green fluorescence and JB 8 showed yellowish green fluorescent pigment (Fig. 2) under UV light. AC 2 and AN 3 were Gram +ve rod shaped whereas isolate AF 4, JB 8 and NP 9 were Gram -ve rod shaped bacteria (Table 4). From morphological and microscopic characters, it was assumed that, AC 2 and AN 3 may belong to family *Bacillaceae* and isolate AF 4, JB 8 and NP 9 may belong to *Pseudomonadaceae*.

Table 4: Fluorescence test and Gram's reaction of efficient antagonistic bacterial isolates.

Isolates	Fluorescence	Shape	Gram's reaction
AC 2	-	Rod	+ve
AN 3	-	Rod	+ve
AF 4	+	Rod	-ve
JB 8	+	Rod	-ve
NP 9	-	Rod	-ve

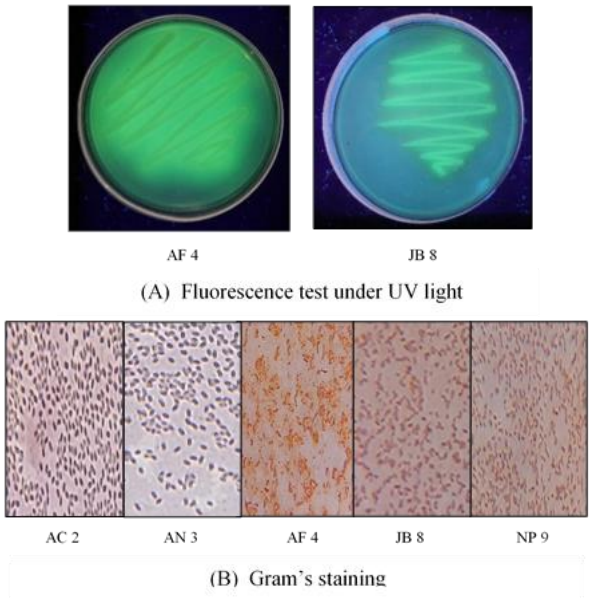


Fig. 2. Morphological characterization of efficient antagonistic bacterial isolates.

F. Biochemical Characterization

HCN Production: Among five isolates, AF 4 showed dark orange colour and NP 9 showed light orange colour after 3 days of incubation (Table 5), these both isolates were found to be positive for HCN production (Fig. 3).

ACC deaminase production: All the isolates were found to grow luxuriously on control plates containing $(NH_4)2SO_4$ as nitrogen source, whereas, exhibited limited growth on plates containing nitrogen free MS media. Moreover, AF 4 and JB 8 showed luxurious growth on plates having ACC as sole source of nitrogen showed their ability to produce enzyme ACC deaminase (Table 5 and Fig. 3).

Cell wall degrading enzymes production: All the five bacterial isolates were capable of producing lipase enzyme and isolate AC 2, AF 4, JB 8 and NP 9 produced protease enzyme. Moreover, only isolate AN 3 and JB 8 were capable of producing chitinase enzyme (Table 5 and Fig. 3).

Table 5: Biochemical characterization of efficient antagonistic bacterial isolates ('+++' strong, '++' moderate, '+' positive, '-' Negative, 'ND' not detected).

Isolates	HCN production	ACC deaminase activity	Cell wall degrading enzymes		
			Lipase	Protease	Chitinase
AC 2	-	-	+	+	ND
AN 3	-	-	+	ND	+
AF 4	+++	+++	+	+	ND
JB 8	-	+++	+	+	+
NP 9	++	-	+	+	ND

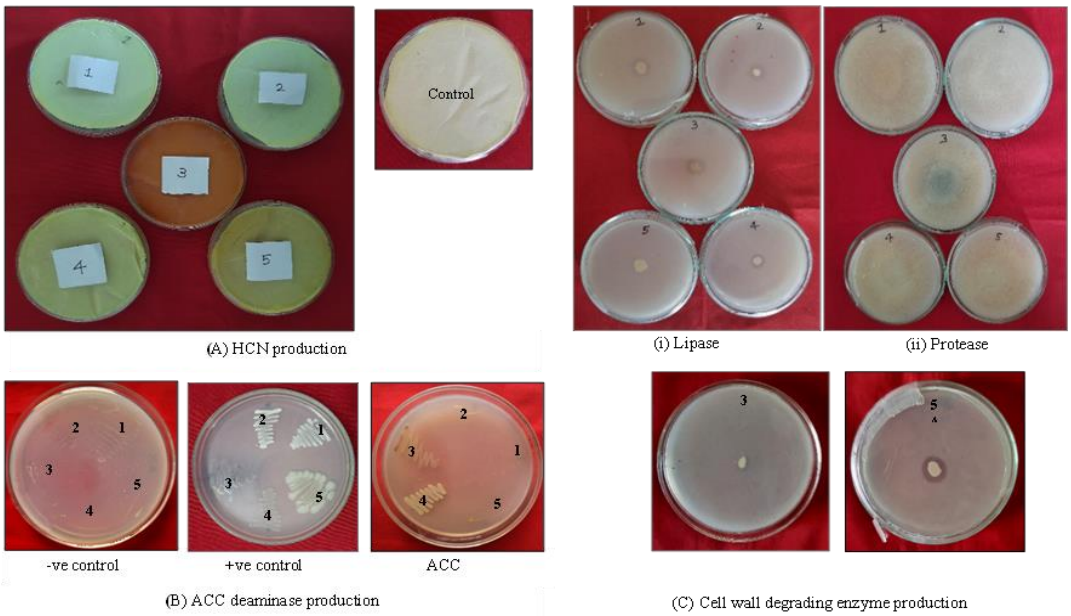


Fig. 3. Biochemical characterization of efficient antagonistic bacterial isolate.

Antibiotic Resistance Profile: Data pertaining to antibiotic resistance profile of efficient antagonistic bacterial isolates is presented in Table 6. All the isolates were highly resistant to Penicillin-G, Carbenicillin, Ampicillin and Spectinomycin. AF 4 was resistant to most of the tested antibiotics except Streptomycin (10 µg/disc), Gentamicin, Polymixin-B, Chloramphenicol and Ciprofloxacin to which it showed susceptibility. On the other hand, JB 8 was also highly resistant to most of the tested antibiotics except Tetracycline, Nalidixic acid, Streptomycin, Kanamycin, Gentamicin, Polymyxin-B, Chloramphenicol and Tetracycline (30 µg/disc). All the isolates were susceptible to Streptomycin (10 µg/ disc), Gentamicin and Polymyxin-B, whereas highly susceptible to Ciprofloxacin.

Table 6: Antibiotic resistance profile of antagonistic bacterial isolates.

Isolates / Antibiotics (µg)	AC 2		AN 3		AF 4		JB 8		NP 9	
	Diameter (mm)	Reaction	Diameter (mm)	Reaction	Diameter (mm)	Reaction	Diameter (mm)	Reaction	Diameter (mm)	Reaction
P- Penicillin-G (10)	0.00	HR	0.00	HR	0.00	HR	0.00	HR	0.00	HR
E- Erythromycin (15)	0.00	HR	37.33	HS	0.00	HR	0.00	HR	20.00	S
RIF- Rifampicin (5)	29.67	HS	12.00	S	0.00	HR	0.00	HR	18.00	S
T or TE- Tetracycline (10)	29.67	HS	37.00	HS	0.00	HR	11.33	S	26.00	HS
NA- Nalidixic acid (30)	30.00	HS	21.33	HS	0.00	HR	12.67	S	12.00	S
S- Streptomycin (10)	13.67	S	13.33	S	11.64	S	12.33	S	16.67	S
K- Kanamycin (30)	18.33	S	18.33	S	0.00	HR	13.00	S	13.33	S
NV- Novobiocin (30)	27.33	HS	20.67	HS	0.00	HR	0.00	HR	18.67	S
G OR GEN- Gentamicin (10)	17.00	S	16.00	S	12.33	S	14.33	S	14.00	S
PB- Polymyxin-B (300)	10.33	S	11.67	S	11.33	S	17.33	S	13.33	S
CB- Carbenicillin (30)	0.00	HR	0.00	HR	0.00	HR	0.00	HR	0.00	HR
VA- Vancomycin (30)	20.00	S	18.67	S	0.00	HR	0.00	HR	12.00	S
AMP- Ampicillin (10)	0.00	HR	0.00	HR	0.00	HR	0.00	HR	0.00	HR
CV- Cephradine (25)	16.33	S	16.67	S	0.00	HR	0.00	HR	31.00	HS
SPT- Spectinomycin (100)	0.00	HR	0.00	HR	0.00	HR	0.00	HR	0.00	HR
C- Chloramphenicol	22.00	HS	20.33	S	10.67	S	18.67	S	0.00	HR
T or TE- Tetracycline (30)	31.33	HS	35.00	HS	0.00	HR	16.33	S	23.33	HS
CIP- Ciprofloxacin (5)	36.00	HS	29.00	HS	22.67	HS	23.00	HS	25.00	HS

G. Molecular Characterization

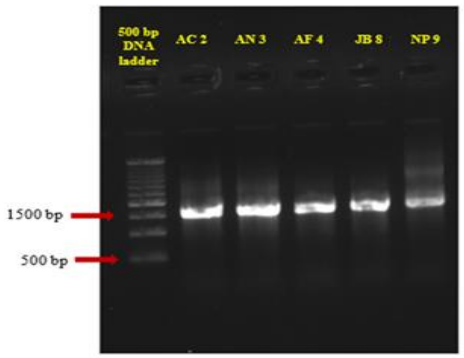


Fig. 4.

PCR and ARDRA products: Approximate products 1500 bp portion of the 16S rDNA gene from the bacterial isolates were amplified using the universal primers 27f and 1495r. They showed successful amplification of targeted amplicon in all bacterial isolates (Fig. 4).

Products of 16S rDNA obtain from different efficient bacterial isolates were subjected to restriction digestion with *Alu* I, *Rsa* I and *Taq* I. The digested products (Fig. 5) showed total of 62 fragments by 3 restriction enzymes. Number of restriction fragments obtained with each enzyme are shown in Table 7.

Fragment analysis: In order to assess the existence of species specificity, restriction patterns were obtained for each of the enzyme utilized and have showed different restriction patterns for the antagonistic isolates. As reported in Table 7, enzyme *Alu* I restriction pattern obtained 21 DNA restriction fragments. Restriction analysis of antagonistic bacterial isolates allowed five species specific profile. Enzyme *Rsa* I obtained 19 restriction fragments of antagonistic bacterial isolates indicating five species specific restriction pattern. While enzyme *Taq* I showed 22 restriction fragments and allowed four specific restriction profiles (Fig. 5).

Table 7: ARDRA pattern of antagonistic bacterial isolates obtained with enzyme *Alu* I, *Rsa* I, *Taq* I.

Sr. No.	Isolate	ARDRA pattern	
		Total number of bands	RE fragment size (bp)
Enzyme *Alu* I			
1.	**AC 2**	4	141, 208, 287, 922
2.	**AN 3**	4	141, 223, 287, 500
3.	**AF 4**	4	147, 199, 244, 450
4.	**JB 8**	4	137, 191, 244, 690
5.	**NP 9**	5	130, 173, 216, 400, 500
Enzyme *Rsa* I			
1.	**AC 2**	2	710, 1338
2.	**AN 3**	3	135, 412, 550
3.	**AF 4**	4	171, 249, 375, 710
4.	**JB 8**	4	171, 197, 375, 1060
5.	**NP 9**	6	156, 178, 216, 375, 432, 530
Enzyme *Taq* I			
1.	**AC 2**	4	144, 219, 375, 526
2.	**AN 3**	4	136, 219, 352, 979
3.	**AF 4**	4	133, 220, 360, 776
4.	**JB 8**	4	125, 187, 360, 827
5.	**NP 9**	6	117, 166, 241, 435, 684, 818

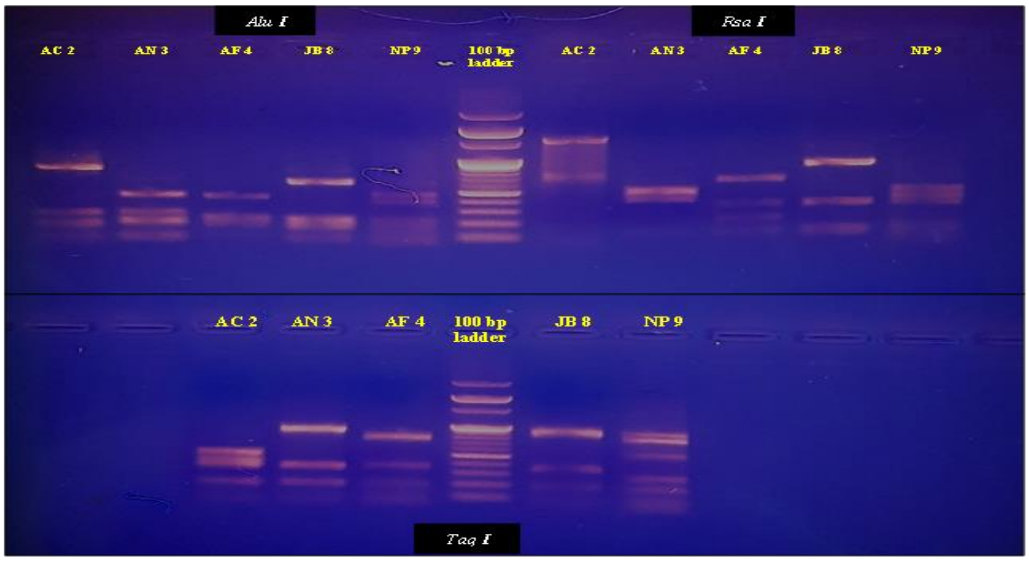


Fig. 5. ARDRA pattern of antagonistic bacterial DNA with *Alu* I, *Rsa* I and *Taq* I enzymes.

Phylogenetic analysis: With *Alu* I enzyme, AC 2 showed 76%, 53%, 53% and 47% similarity (Table 8) with AN 3, AF 4, JB 8 and NP 9, respectively. Whereas AN 3 showed 53%, 53% and 59% similarity with isolate AF 4, JB 8 and NP 9, respectively. AF 4 showed 65% similarity with JB 8 and JB 8 showed 47% similarity with NP 9 (Fig. 6). With enzyme *Rsa* I, AC 2 showed 67%, 73%, 60% and 47% similarity with isolate AN 3, AF 4, JB 8 and NP 9, respectively. Whereas AN 3 showed 53%, 53% and 40% similarity with isolate AF 4, JB 8 and NP 9, respectively. AF 4 showed 73% similarity with JB 8 and 47% similarity with NP 9. JB 8 showed 47% similarity with NP 9 (Table 9 and Fig. 6). With enzyme *Taq* I, AC 2 showed 70%, 60%, 60% and 50% similarity with isolate AN 3, AF 4, JB 8 and NP 9, respectively. Whereas AN 3 showed 60%, 60% and 50% with isolate AF 4, JB 8 and NP 9. AF 4 showed 70% similarity with JB 8 and 50% similarity with NP 9, JB 8 showed 50% similarity with NP 9 (Table 10 and Fig. 6).

Table 8: The pair-wise correlation generated based on the restriction fragment for *Alu* I.

Isolate	AC 2	AN 3	AF 4	JB 8	NP 9
AC 2	1.00				
AN 3	0.76	1.00			
AF 4	0.53	0.53	1.00		
JB 8	0.53	0.53	0.65	1.00	
NP 9	0.47	0.59	0.47	0.47	1.00

Table 9: The pair-wise correlation generated based on the restriction fragment for *Rsa* I.

Isolate	AC 2	AN 3	AF 4	JB 8	NP 9
AC 2	1.00				
AN 3	0.67	1.00			
AF 4	0.73	0.53	1.00		
JB 8	0.60	0.53	0.73	1.00	
NP 9	0.47	0.40	0.47	0.47	1.00

Table 10: The pair-wise correlation generated based on the restriction fragment for *Taq* I.

Isolate	AC 2	AN 3	AF 4	JB 8	NP 9
AC 2	1.00				
AN 3	0.70	1.00			
AF 4	0.60	0.60	1.00		
JB 8	0.60	0.60	0.70	1.00	
NP 9	0.50	0.50	0.50	0.50	1.00

Pooled analysis: Pooled analysis of ARDRA for antagonistic bacterial isolates is represented by dendrogram (Fig. 6). AC 2 showed 71%, 61%, 58% and 48% similarity with isolate AN 3, AF 4, JB 8 and NP 9, respectively. Whereas AN 3 showed 66%, 56% and 50% similarity with isolate AF 4, JB 8 and NP 9. AF 4 showed 69% similarity with JB 8 and 48% similarity with NP 9. JB 8 showed 48% similarity with NP 9 (Table 11).

Overall, ARDRA results indicated that isolates AC 2 and AN 3 closely related but may be different at species level. Similarly, AF 4 and JB 8 were closely related but may be different at species level. While NP 9 showed 48-50% similarity with other isolates indicating diversity at genus level (Fig. 6).

Table 11: Pooled pair-wise correlation between bacterial isolates.

Isolate	AC 2	AN 3	AF 4	JB 8	NP 9
AC 2	1.00				
AN 3	0.71	1.00			
AF 4	0.61	0.66	1.00		
JB 8	0.58	0.56	0.69	1.00	
NP 9	0.48	0.50	0.48	0.48	1.00

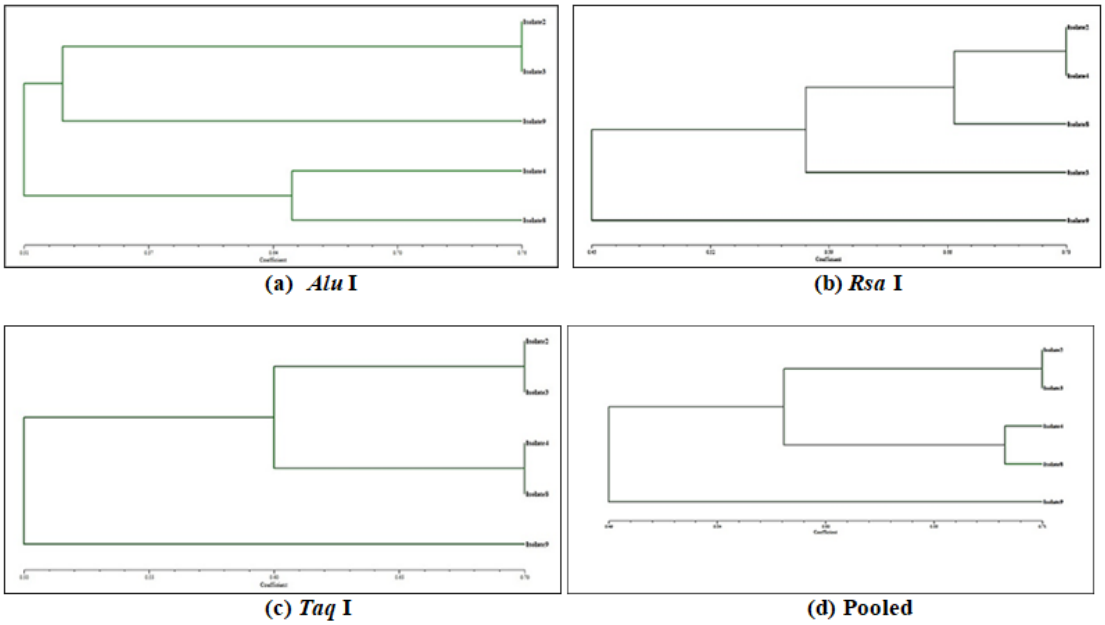


Fig. 6. Dendrogram of antagonistic bacterial isolates based on ARDRA profile of (a) *Alu* I, (b) *Rsa* I, (c) *Taq* I and (d) pooled.

Bacterial identification and 16S rDNA sequence analysis: 16S rDNA partial gene sequence of ~ 1500 bp was carried out (with technical support of Eurofins Genomics, Bengaluru) and the output data were stored in FASTA format as described below (Table 12). AC 2 was identified as *Bacillus licheniformis* with 99.61 per cent similarity and 99 per cent query coverage to *B. licheniformis* strain ATCC 14580 (Table 12). Additionally, the phylogenetic position of the isolate was also worked out within the available database of NCBI. The phylogenetic tree indicated, AC 2 belongs to Firmicutes and grouped with another *Bacillus* spp. AN 3 was identified as *Bacillus stratosphericus* showed 99.92 per cent identity with *B. stratosphericus* strain 41KF2a with 100 per cent query coverage (Table 12) which confirms the AN 3 belongs to *Bacillus* spp.

AF 4 was identified as *Pseudomonas aeruginosa* with 99.29 per cent similarity and 98 per cent query coverage to *Pseudomonas aeruginosa* strain ATCC 10145 (Table 12). Phylogenetic tree constructed showed that the second cluster have also indicated its close relation with other members of g- Proteobacteria group.

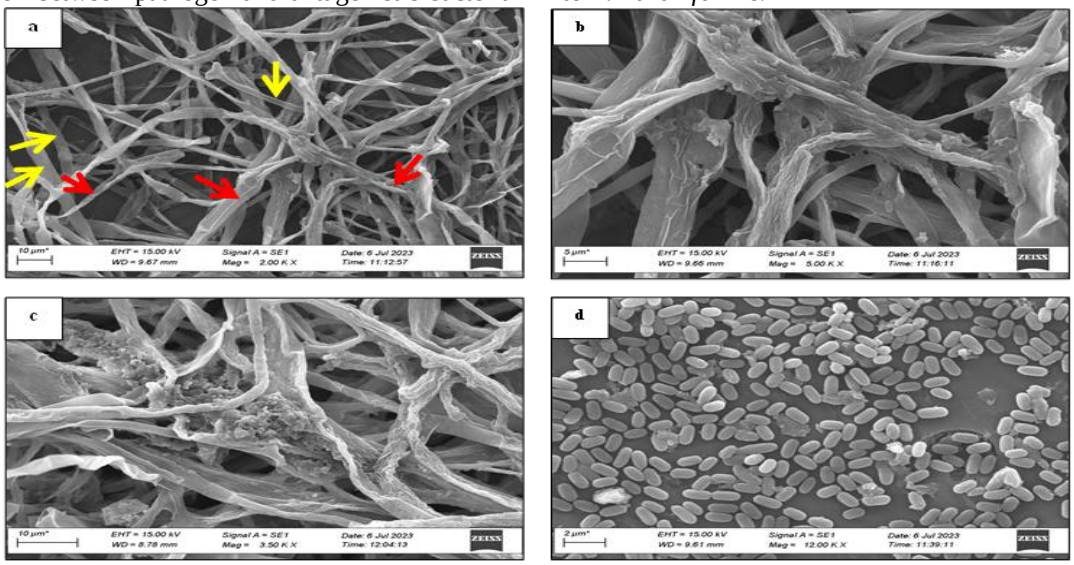
JB 8 showed about 99.44 per cent similarity and 100 per cent query coverage with *Pseudomonas paracarnis* strain V5/DAB/2/5 indicating JB 8 may the native species of *P. paracarnis* strain (Table 12). The phylogenetic tree constructed showed one major cluster with another *Pseudomonas* spp. NP 9 was identified as *Stenotrophomonas* spp. Showed 94.11 per cent identity with *Stenotrophomonas lactitubi* strain M15 with 100 per cent query coverage (Table 12). Which confirms the NP 9 belongs to *Stenotrophomonas* genus but the species was not confirmed.

Based on the above results and analysis, isolate AC 2 was identified and named as *Bacillus licheniformis* AAU BCM 1 (NCBI Accn no: OR002034), isolate AN 3 as *Bacillus stratosphericus* AAU BCM 2 (NCBI Accn no: OQ998805), isolate AF 4 as *Pseudomonas aeruginosa* AAU BCM 3 (NCBI Accn no: OQ998804), isolate JB 8 as *Pseudomonas azotoformans* AAU BCM 4 (NCBI Accn no: OQ998897) and NP 9 as *Stenotrophomonas* sp. AAU BCM 5 (NCBI Accn no: OQ998899).

Table 12: Identification of rhizospheric antagonistic bacterial isolates by 16S rDNA sequencing.

Isolate	Length of 16S rDNA gene sequenced	Gene Bank accession no.	Most closely related organisms*			
			Species	Accession description	% Gene identity	% Query coverage
AC 2	1295	OR002034	*Bacillus licheniformis* strain ATCC 14580	NR_074923.1	99.61	99
AN 3	1306	OQ998805	*Bacillus stratosphericus* strain 41KF2a	NR_118441.1	99.92	100
AF 4	1283	OQ998804	*Pseudomonas aeruginosa* strain ATCC 10145	NR_114471.1	99.29	98
JB 8	1249	OQ998897	*Pseudomonas paracarnis* strain V5/DAB/2/5	NR_178976.1	99.44	100
NP 9	629	OQ998899	*Stenotrophomonas lactitubi* strain M15	NR_179509.1	94.11	100

Scanning electron microscopy: As Scanning Electron Microscopy (SEM) photographs of dual culture method of *B. licheniformis* against *M. phaseolina* under in-vitro conditions presented in Fig. 7, the hypha of pathogen *M. phaseolina* was normal when there was no interaction between pathogen and antagonistic bacteria *B. licheniformis* (AC 2). Whereas, in case of interaction between pathogen and bacteria there was shrinkage of the hyphae can be seen. Dense proliferation of bacteria over the mycelial surface can be seen at the interaction zone, that clearly indicates the hyphal destruction due to *B. licheniformis.*



(a) is showing normal hyphae of *M. phaseolina* which is not affected by antagonistic bacteria is showing shriveled hyphae of *M. phaseolina* which is affected by antagonistic bacteria; (b) Hyphal destruction due to *B. licheniformis* (c) Dense proliferation of bacteria over the mycelial surface; (d) Bacterial cells of *B. licheniformis.*

Fig. 7. Scanning electron micrographs of dual culture assay of *B. licheniformis* against *M. phaseolina* in-vitro.

CONCLUSIONS

The study successfully identified and characterized five native rhizospheric bacterial isolates with potent antagonistic activity against *Macrophomina phaseolina*, the causal agent of root rot in black gram. Dual culture assays confirmed their efficacy, with growth inhibition nearing 70%. The isolates, identified through 16S rDNA sequencing and ARDRA, belong to diverse genera—*Bacillus*, *Pseudomonas*, and *Stenotrophomonas*. Their ability to produce antifungal enzymes, HCN, and ACC deaminase suggests a multifaceted mechanism of pathogen inhibition and stress alleviation in plants. The antibiotic resistance exhibited by these isolates may support their survival and colonization in the rhizosphere, enhancing their utility as biocontrol inoculants. The SEM images further corroborated the antagonistic interactions by showing physical damage to *M. phaseolina* hyphae. These findings underscore the potential of leveraging indigenous rhizobacteria for eco-friendly and effective disease management in black gram cultivation.

FUTURE SCOPE

The selected antagonistic bacterial isolates show strong potential for development as biocontrol agents against *M. phaseolina* in black gram. Future work should focus on validating their efficacy through greenhouse and field trials, developing stable bioformulations, and exploring their additional plant growth-promoting traits. Genomic studies could further clarify the molecular mechanisms behind their antifungal and stress-alleviating properties, supporting their broader application in sustainable agriculture.

Acknowledgement. The authors are grateful to the Department of Plant Pathology and Department of Agricultural Microbiology, B.A. College of Agriculture, Anand Agricultural University, Anand for providing all the resources in this research work.

Conflict of Interest. None.

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How to cite this article: Prajapati M.D., Puja Pandey, Yogeshvari K. Jhala, Hardik H. Patel and Hiren K. Patel (2025). Exploring Higher Root Rhizospheric Soil as a Source of Potential Bacterial Antagonist Against Root Rot Fungi *Macrophomina phaseolina* Infecting Black Gram. *Biological Forum*, *17*(7): 116-127.