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Evaluation of Antagonistic Activity of Phylloplane Bacteria Against Finger Millet Blast Disease caused by *Magnaporthe grisea*

G. Sobanbabu[1*], M. Theradimani[2], S. Thiruvudainambi[3], C Vannirajan[4], S. Rajavel[5], K. Kalpana[6], S. Sindhu[7] and S. Brindhadevi[1]

[1]Ph.D. Scholar, Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, (Tamil Nadu), India.
[2]Professor and Head, Department of Plant Pathology, Agricultural College and Research Institute, Madurai-625104, (Tamil Nadu), India.
[3]Professor, Department of Plant Pathology, Agricultural College and Research Institute, Madurai-625104, (Tamil Nadu), India.
[4]Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, (Tamil Nadu), India.
[5]Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, (Tamil Nadu), India.
[6]Assistant Professor, Department of Plant Pathology, Agricultural College and Research Institute, Madurai-625104, (Tamil Nadu), India.
[7]PG Scholar, Department of Plant Pathology, Agricultural College and Research Institute, Madurai-625104, (Tamil Nadu), India.

(Corresponding author: G. Sobanbabu)*
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ABSTRACT: Finger millet (*Eleusine coracana*) is the most nutritious crop and it has severely affected by blast disease is caused by *Magnaporthe grisea*. The disease is effectively managed by fungicides and some resistant varieties. The resistant varieties are affecting by pathogen due to agroclimatic changes and the fungicides are hazardous to ecosystem. Biocontrol agent is an alternative method for managing the blast disease and it involve directly or indirectly inhibits the growth of the pathogen. Totally thirty bacterial isolates were isolated on phylloplane region of finger millet crop. Out of thirty bacterial isolates, four isolates were found to be effective antagonists *viz.*, DPB1, KB1, KP1 and DPP1. Among them, KB1 isolate showed the maximum inhibition of mycelial growth of *M. grisea* (62.87%) followed by DPB1 isolate (54.54 %). On biochemical screening, *Bacillus* spp. were identified by gram staining, starch hydrolysis and protease production tests and *Pseudomonas* spp. were identified by gram staining, phosphate solubilization and siderophore production test. The specific species were molecularly characterized through 16S rRNA gene sequencing. From BLAST NCBI database, the isolates were identified as *Bacillus subtilis* (DPB1), *Bacillus cereus* (KB1), *Pseudomonas aeruginosa* 1 (KP1) and *Pseudomonas aeruginosa* 2 (DPP1). The present investigation, we have identified the effective antagonists against blast pathogen such as, *Bacillus subtilis* (DPB1) and *Bacillus cereus* (KB1) isolates and the isolates species were confirmed through molecular characterization.

Keywords: Finger millet, Blast, Antagonists, Biochemical, Molecular characterization.

INTRODUCTION

Finger millet is accounted for its nutraceutical values and come up with the diversified climate and ranks fourth position in production over the world. It is the most climate resilient crop with long storage period. They are often prone to biotic stress such as pests and diseases (Mbinda *et al*., 2020). Among the 20 diseases, ragi blast disease threatens the cultivation of ragi throughout the world. Finger millet blast is caused by *Magnaporthe grisea* belongs to the family *Ascomycota. Magnaporthe* is the most destructive pathogen can attack all the stages of ragi crop. Thereby, it brings significant yield loss up to 100 % (Sekar *et al*., 2018). However, chemical fungicides can be opted for disease management but its continuous usage leads to the

residual effect and hazard to the environment with the evolution of fungicidal resistance towards the chemical. Nowadays, consumers hugely demand for safe and eco-friendly food products. The biocontrol agents will pave the alternative way to the sustainable agriculture and will reduce the usage of fungicides in turn can reduce the fungicide contamination and simultaneously promotes the growth of plants (Chakraborty *et al*., 2021).

The PGPR are directly or indirectly interact with plants and they elicit biochemical and molecular defense response within the plants. They are secreting some compounds which inhibit growth and reproduction of the pathogen (Martins *et al*., 2020). In plants, the beneficial microorganisms are present in both ephiphyte and endophyte region and they are collectively called as PGPR (Aeron *et al*., 2020). Historically, numerous potential antagonists have been isolated from rhizosphere region and have been commercialized for growth enhancement, seed emergence and disease management. Blast disease causes infection on the aerial parts of finger millet crop. Then, it would be wise to find suitable phylloplane colonizing antagonistic microbes that could survive in the dynamic environmental conditions of phylloplane region and also suppress the pathogen (Suguna *et al*., 2020).

Usage of phylloplane microbes can be one of the approaches to manage the plant diseases. These microbes can elicit the induced systemic resistance which prevents the host from the diseases (Harish *et al*., 2007). Phylloplane refers to the surface of the leaves which is mainly habituated predominately by bacteria followed by archaebacteria, fungi and yeasts. Various Phylloplane microbes such as *Pseudomonas, Psychrobacter, Streptomyces, Bacillus, Acinetobacter, Microbacterium, Kineococcus* etc were identified for the plant growth promotion (Batool *et al*., 2016). Phylloplane microbes have direct detrimental effect against spore producing pathogenic microbes. They can also be used as bioprotectants by producing chemical substances such as siderophore, antibiotics etc. They can also trigger the defense related enzymes such as phenylalanine ammonia lyase, polyphenol peroxidase and peroxidase which resulted due to induced systemic resistance in host plants (Stevens *et al*., 2021). Application of phylloplane microbes leads to enhanced crop growth, yield and improved soil health. They can also be able to produce plant growth hormones there by can fix and mobilize the nutrients to the soil. Thereby host plants can easily derive the nutrients from the soil through the root hairs. Also, the phylloplane microbes can compete for the space and nutrients (Goswami *et al*., 2021).

In this study, the phylloplane bacterial antagonists were isolated from the Finger millet healthy leaves were evaluated for their antagonistic activity, Biochemical characterization and Molecular characterization under *in vitro* for *Magnaporthe grisea*.

MATERIALS AND METHODS

A. Isolation of phylloplane bacterial antagonists from finger millet crop

The healthy leaves were collected from finger millet crop and they were individually washed by transferring each leaf one by one in a conical flask containing 100 ml of sterile water with 0.01% Tween 20 (50 micro litre/100 millilitre). One millilitre of the suspension was taken from the 10 ml of the stock and added to the 9 ml sterile water containing test tubes and noted as first dilution. Similarly, the aliquot was serially diluted up to seven dilutions. For obtaining bacterial culture, one ml of suspension from the fifth and sixth dilutions were plated on Nutrient Agar. Then the obtained culture isolates were maintained on nutrient agar slants and stored at 4°C for further studies.

B. Effect of phylloplane bacterial antagonists against M. grisea under in vitro

The pathogen was cultured on Potato dextrose agar medium and the phylloplane bacterial isolates were multiplied on nutrient agar medium respectively. The antagonistic effect against the pathogen was tested by dual culture technique (Dennis and Webster, 1971). A nine millimetre actively growing eight days old culture disc of *M. grisea* was placed at one side of the Petri plate onto PDA medium. Antagonistic bacterial isolate was streaked at the opposite side of the Petri plate at one day and three days after *M. grisea* inoculation respectively. Three replications along with the suitable control without antagonist were maintained. The plates were incubated in an inverted position at room temperature (25 ± 2°C) for 7 days. The linear growth of the pathogen was measured. The growth inhibition per cent was calculated using the following formula.

$$PI = \frac{Dc - dt}{dc} \times 100$$

Dc = Average diameter of fungal growth (mm) in control

Dt = Average diameter of fungal growth (mm) in treatment

PI = Per cent inhibition over control

C. Biochemical tests for identification of phylloplane bacterial antagonists

Gram's staining. A thin smear of bacterial culture was made in the glass slide, air dried, heat fixed and coated with crystal violet for 30 seconds and followed by Gram's iodine for 30 seconds. The organism was decolorized with 95% ethyl alcohol. Then safranin was applied to the smears for 30 seconds and washed, blot dried and then examined microscopically using oil-immersion at 100 X magnification.

Starch hydrolysis. The nutrient agar containing 0.2 per cent soluble starch was used. The test cultures were streaked on the medium and incubated at 28°C for 3 days. Starch hydrolysis was tested after 3 days of incubation by flooding the agar surface with Lugol's

iodine solution. A clear colourless zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction.

Siderophore production assay. The universal CAS (Chromazurol S) assay was modified to test the siderophore production of bacterial strains. 1. Blue dye solution preparation: Solution 1: 60 mg CAS dissolved in 50 ml distilled water. Solution 2: 27 mg of $FeCl_3.6H_2O$ is dissolved in 10 ml of 10 mM HCl. Solution 3: 73 mg of HDTMA (CTAB) was dissolved in 40 ml of water.

Then, 1 ml of solution 1 was mixed with 9 ml of solution 2. This entire mixture was mixed with 40 ml of solution 3 and then the medium became blue. Later it was autoclaved and stored. The Mixture 1 solution was prepared by adding 100 ml of MM9 solution (15g KH_2PO_4, 25g NaCl, and 50g NH_4Cl in 500 ml of dd H_2O), 2 g of glucose, 3 g of casamino and agar 20g. The volume was made up to 1000 ml and then autoclaved. After autoclaving, 100 ml of blue dye was poured into 900 ml of the mixture solution. Then the medium was poured onto plates. After solidification, two days old actively growing bacterial antagonists were streaked on the medium and incubated the plate at 30°C. After 3 days of incubation, yellow hollow zone around the bacterial streaked line indicates positive reaction for siderophore production.

Protease production. Protease positive strains were screened for proteolytic activity on casein agar plates. Plates were spot inoculated and incubated at 28°C for 24 to 48 h. After incubation, the positive cultures were detected by the presence of clear zones around the colonies.

Phosphate solubilisation. A single bacterial colony was chosen and streaked onto the Pikovskaya's agar medium (Pikovskaya, 1948). Positive reaction indicates the formation of clear zone around the colony after the third of incubation at the temperature of 30°C.

D. Molecular characterization of phylloplane bacterial antagonists by 16S rRNA gene sequencing

Isolation of total genomic DNA from bacteria. The standard protocol of Cetyl trimethyl ammonium bromide (CTAB) method was used to isolate the total genomic DNA from potential antagonistic bacterial isolates

Two millilitres of actively grown broth culture was taken in 2 ml centrifuge tube and centrifuged at 6,000 rpm for 5 mins at 4°C. The supernatant was removed and the pellet was suspended in 1 ml TE buffer. To that, 0.5 ml of 1-butanol was added and vortexed well to mix the cells thoroughly (to remove extra cellular materials) and centrifuged at 6000 rpm for 5 min at 4°C.

The supernatant (both supernatant layer and aqueous layer) was discarded and the pellet was re-suspended in 2 ml of TE buffer and centrifuged at 6,000 rpm at 4°C for 5 min to remove all traces of butanol. Again, the pellet was re-suspended in 1ml TE buffer. To that, 100 µl of lysozyme (10 mg ml^{-1} freshly prepared) was added and incubated at room temperature for 5 min. After incubation, 100 µl of 10 % SDS and 25 µl of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for one hour. To the above mixture, 200 µl of 5M NaCl was added and mixed well. To this mixture, 150 µl of CTAB solution was added, mixed well and incubated at 65°C for 10 min. The mixture was extracted with 1ml of phenol: chloroform mixture in the ratio of 25:24, mixed well and centrifuged at 14000 rpm for 15 min at 4°C. The aqueous layer was transferred carefully to a 2 ml micro-centrifuge tube and DNA was precipitated by adding equal volume of ice cold iso-propanol by incubating overnight at - 20°C. The DNA was pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was washed with 70 per cent ethanol for 2 times and dried under vacuum or kept on water bath at 50-55°C for 10 mins and re-suspended in 50 µl of TE buffer. The DNA was stored at -20°C for further use. The bacterial genome DNA was verified using 1% Agarose gel electrophoresis method.

16S rDNA sequencing of phylloplane bacterial antagonists (Isik *et al.*, 2014)

A PCR was performed in a total volume of 50 µl using Emerald Amp® GT PCR master mix using genomic DNA of phylloplane bacteria as a template. The small sub unit 16S regions were amplified with universal primers. The primers were 27F + 1492r PCR conditions of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing temperature at 50°C for 30 sec and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The reaction was carried out in Eppendorf master cycler gradient PCR machine. The PCR products were resolved by electrophoresis in 1% agarose gel. The PCR products were purified using FavorPrep GEL/ PCR purification kit and sequenced at Bioserve Biotechnologies India Pvt, Ltd. Hyderabad.

The Primers sequence used for amplification of 16S rRNA region were,

27f - 5′ AGAGTTTGATCTGGCTCAG 3′ forward primer)

1492r - 5′ TACGGYTACCTTGTTACGACT 3′ (reverse primer)

Sequencing of 16S rDNA and identification of phylloplane antagonistic bacteria by bioinformatics analysis. The obtained DNA sequences were input sequence (Query sequence) in nucleotide blast analysis program at NCBI database. The output data retrieved from the bioinformatics were analysed and the organism showing major score was considered as the closely related species to the test antagonistic bacteria used in the study.

E. Statistical Analysis

The mean difference between each treatment was found by using ANOVA and the mean was compared with Duncan's Multiple Range Test by using SPSS 16.0 software.

RESULTS AND DISCUSSION

A. Isolation of phylloplane bacterial antagonist from healthy leaves of Finger millet crop

Approximately, thirty phylloplane bacterial antagonists were isolated from the healthy leaves of finger millet. And these thirty isolates were tested for their antagonistic activity against blast pathogen, *M. grisea* by following dual culture assay and from the dual culture assay, the virulent antagonists were identified and selected for biochemical and molecular characterization to identify them at genus and species level. Akter *et al.*, (2014) isolated totally 325 phylloplane microbes from the *Rhizoctonia* infected and healthy rice leaves and out of 325, 14 were found to be having antagonist on preliminary screening against the sheath blight pathogen of rice. These isolates were further subjected to dual culture assay against *Rhizoctonia solani* and tested for their metabolite activity and further they were identified at genus and species level by biochemical and molecular characterization.

B. Effect of Phylloplane bacteria against the mycelial growth of M. grisea in vitro

In vitro antagonistic effects of bacterial antagonist were tested against *M. grisea*. Among the thirty isolates, four isolates exhibited the antagonistic activity against the ragi blast pathogen and were given with the isolates code. The cultural and morphological characters of the virulent isolates were observed (Table 1, Fig 1). Among the four phylloplane bacteria, KB1 isolate showed the maximum growth reduction of *M. grisea* by 62.87 per cent over control that was followed by DPB1 isolate which recorded 54.54 per cent over control. KP1 isolate showed 45.45 per cent over control. The minimum growth reduction of 33.71 per cent was observed in DPP1 isolate (Table 2, Fig 2). Similar study revealed that the phylloplane microbes from the paddy crop showed antagonistic activity towards the mycelial growth of sheath rot pathogen. Raja and Mahalakshmi (2014), isolated nine phylloplane microbes from the rice crop and their antagonistic activity were tested by dual culture assay. The results obtained from the dual culture showed that maximum mycelial growth inhibition was recorded with *Pseudomonas fluorescens* followed by *Bacillus subtilis*. Chakraborty *et al*., (2021) studied the antagonistic activity of several microbes and reported that *Bacillus cereus* II 14, *B. firmus* E65 and *Pseudomonas aeruginosa* C32b inhibited the mycelial growth of *Magnaporthe grisea* under *in vitro.*

Table 1: Morphological characters of the virulent isolates of phylloplane bacterial antagonists.

Sr. No.	Virulent isolates	Cultural and morphological characters		
		Colony colour	Colony form	Colony margin
1.	DPB1	Creamy	Circular, smooth and powdery	Deeply serrated with wavy edge
2.	KB1	Dull White	Irregular, smooth and powdery	Deeply serrated with wavy edge
3.	KP1	Yellowish green	Circular, smooth and slimy	Fine wavy margin
4.	DPP1	Yellowish green	Circular, smooth and slimy	Fine wavy margin

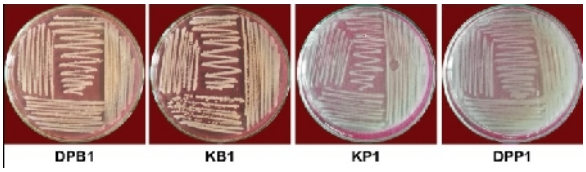


Fig. 1. Morphological characters of the virulent isolates of phylloplane bacterial antagonists.

Table 2: *In vitro* antagonism of Phylloplane bacterial antagonists against mycelial growth of *M. grisea.*

Sr. No.	Virulent isolates	Mycelial growth (mm)*	Percent Inhibition over control (%)
1.	DPB1	40.00[b]	54.54
2.	KB1	32.66[a]	62.87
3.	KP1	48.00[c]	45.45
4.	DPP1	58.33[d]	33.71
5.	Control	88.00[e]	

*Mean of three replications

The treatment means are compared using Duncan's Multiple Range Test (DMRT)

In a column, mean values followed by a common letter (s) are not significantly different (P=0.05)

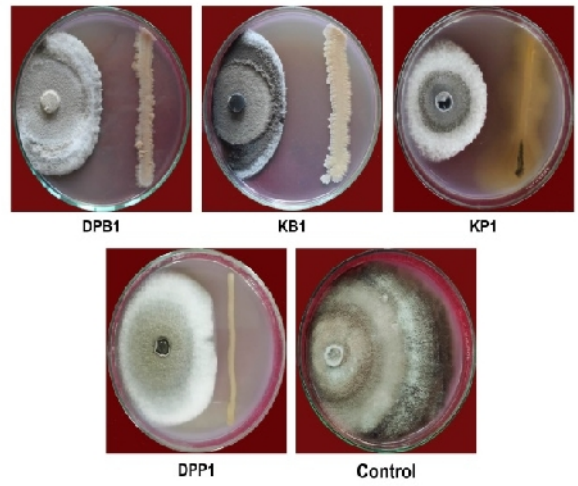
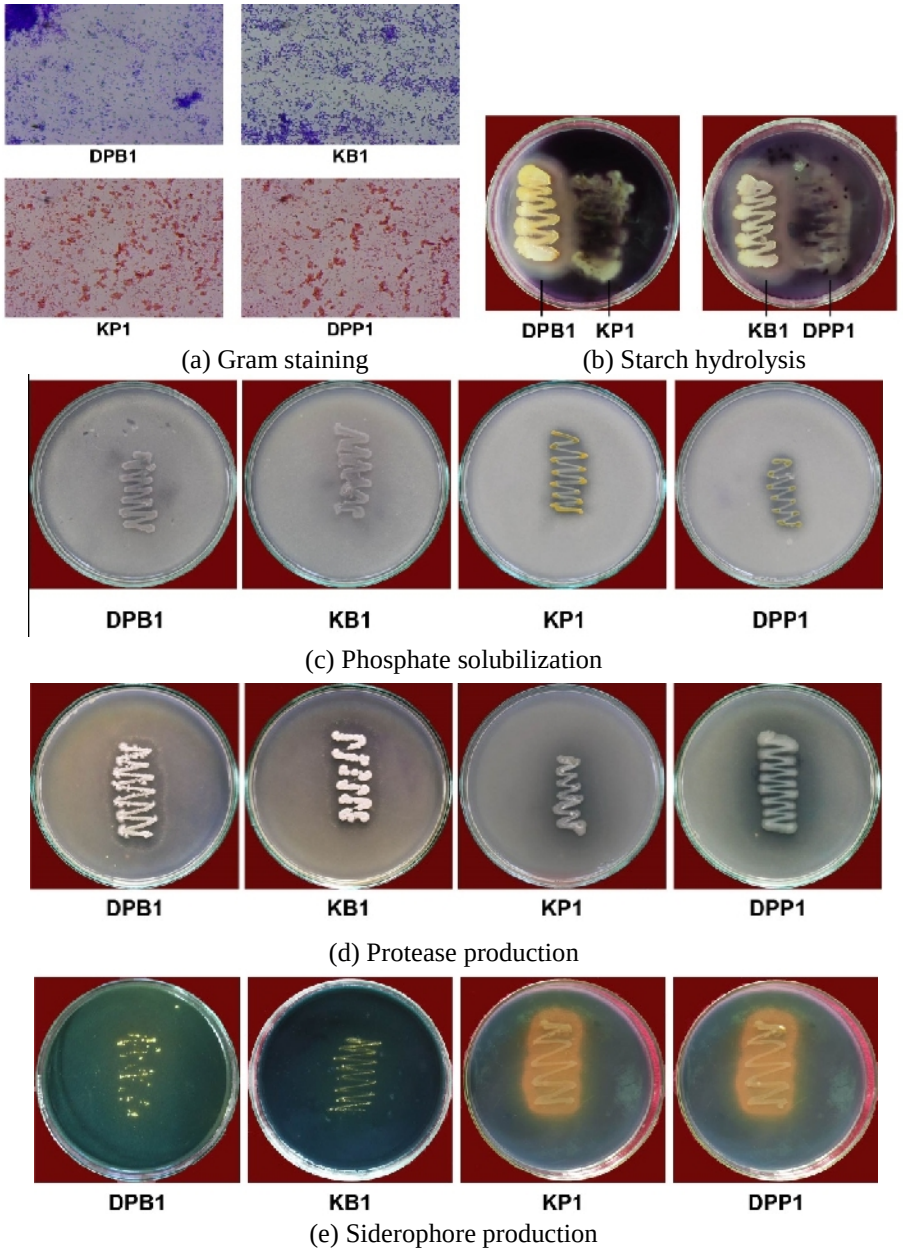


Fig. 2. Effect of Phylloplane bacteria against the mycelial growth of *M. grisea in vitro.*

C. Biochemical tests for identification of phylloplane bacterial antagonist

Among the four virulent isolates *viz*., DPB 1 and KB 1 were found to be gram positive whereas KP 1 and DPP 1 were found to be gram negative. Further, DPB 1 and KB 1 isolates were able to hydrolyse the starch which in turn produced the clear zone around the bacterial colony but it could not solubilize the phosphate present in Pikovskaya's agar medium Whereas KP 1 and DPP 1 isolates were not observed for clear zone, indicated the absence of starch hydrolysis but it could able to solubilize the phosphate in Pikovskaya's agar medium. All the four isolates were noted for proteolytic activity as they produced clear zone around the bacterial colony on casein agar. And also, they were found to be producer of siderophore (iron chelating agent which is orange in colour). Among them KP 1 and DPP 1 isolates produced siderophore maximum intensity compared to DPB 1 and KB 1 isolates. Thus, from the biochemical characterization the isolated phylloplane microbes may be suspected as *Bacillus* (DPB 1 and KB 1) and *Pseudomonas* sp. (KP 1 and DPP 1) (Table 3, Fig. 3a, b, c, d, e). Martins *et al*., (2020) characterized six bacterial isolates from both phylloplane and rhizosphere regions and subjected to different biochemical tests. From the biochemical screening, they distinguished the bacterial isolates into gram positive and gram negative, siderophore producers and non-producers and molecularly confirmed to identify the genus and species.



(a) Gram staining (b) Starch hydrolysis

(c) Phosphate solubilization

(d) Protease production

(e) Siderophore production

Fig. 3. Biochemical tests for identification of phylloplane bacterial antagonist.

Table 3: Biochemical tests for the identification of Phylloplane bacterial antagonists.

Sr. No.	Isolates	Biochemical tests				
		Gram staining	Starch hydrolysis	Siderophore Production	Protease utilization	Phosphate utilization
1.	DPB1	+	+	+	+	-
2.	KB1	+	+	+	+	-
3.	KP1	-	-	+	+	+
4.	DPP1	-	-	+	+	+

D. Molecular characterization of phylloplane bacterial antagonists

16S rDNA sequence analysis is one of the commonly used molecular methods for the identification of bacteria at species level. 16S rRNA from four virulent isolates *viz*., DPB 1, KB 1, KP 1 and DPP 1 were isolated using CTAB method. Single band of intact genomic DNA was visualized on the agarose gel. 16S rRNA region of these bacterial isolates was amplified with primer pairs 27f and 1492r using a thermo cycler and the products produced were visualized as a single band in agarose gel strained with ethidium bromide. The size of the PCR fragments was approximately 1492 bp length for primer pair 27f and 1492r. The PCR products were sequenced at Bioserve Biotechnologies India Pvt, Ltd. The full length 16S rRNA sequences obtained for each bacterial species were BLAST searched in the database of National Centre for Biotechnology Information (NCBI). Four virulent isolates were molecularly identified at the species level as *Bacillus subtilis* (DPB1), *Bacillus cereus* (KB1) and *Pseudomonas aeruginosa* (KP 1 and DPP 1) (Table 4, Fig. 4). Similarly, Batool *et al*., (2016) isolated the phylloplane associated bacteria from the superior wheat cultivars and molecularly identified the best plant growth promoting isolates through 16s rRNA gene sequencing method. Martins *et al*., (2020) isolated six bacterial isolates from the rice crop and given with the isolate code *viz*., BRM 32109, BRM 32110, BRM 32111, BRM 32112, BRM 32113, and BRM 32114 and they were initially identified through morphologically and biochemically. Further they were molecularly characterized with partial sequencing of 16S rRNA and they were identified as *Bacillus* sp. (BRM 32109, BRM 32110), *Pseudomonas fluorescens* (BRM 32111), *Pseudomonas* sp. (BRM 32112), *Burkholderia* sp. (BRM 32113) and *Serratia* sp. (BRM 32114).

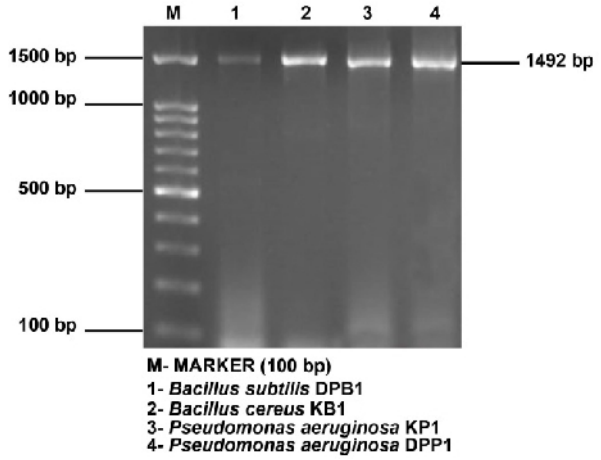


Fig. 4. Molecular characterization of the Phylloplane bacterial antagonists.

Table 4: Molecular characterization of the Phylloplane bacterial antagonists.

Sr. No.	Virulent isolates of Phylloplane bacteria	Identification at species level	Accession number
1.	DPB1	*Bacillus subtilis*	MZ348923
2.	KB1	*Bacillus cereus*	MZ314744
3.	KP1	*Pseudomonas aeruginosa*	OL831015
4.	DPP1	*Pseudomonas aeruginosa*	OL831145

CONCLUSION

In this study, we have isolate totally 30 phylloplane bacteria and to find four effective antagonistic bacteria by dual culture technique. The effective antagonistic bacteria were confirmed through biochemical and molecular characterization (*Bacillus subtilis* (DPB1), *Bacillus cereus* (KB1), *Pseudomonas aeruginosa* 1 (KP1) and *Pseudomonas aeruginosa* 2 (DPP1). The biocontrol agents are alternative method for management of diseases and it is free from hazardous for environment and human health.

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Conflict of Interest. None.

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