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Isolation and molecular identification of potential bacterial antagonist for the management of brown leaf spot of mulberry

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Abstract

Fifty bacterial isolates were obtained in axenic form from the phylloplane of mulberry variety S1635, of which five were found to be antagonistic to *Paramyrothecium roridum* (Syn: *Myrothecium roridum* Tode ex. Fr.) causing brown leaf spot of mulberry. Further evaluation revealed that all the five bacterial isolates significantly reduced the radial growth of brown leaf spot pathogen. Gram positive, rod shaped bacterial isolate MP-18-5 was found to be most effective against the pathogen exhibiting 62.0% radial mycelial growth inhibition. Based on 16S rRNA gene sequence analysis, strain MP-18-5 was identified as belonging to the genus *Bacillus* with *Bacillus zhangzhouensis* DW5-4T (sequence similarity value of 97.79%) as its closest phylogenetic neighbour.

Keywords: Antagonist, biocontrol, brown leaf spot, dual culture and mulberry

Introduction

Mulberry (*Morus* spp.) belonging to the family Moraceae is an economically important perennial plant cultivated under wide range of agro climatic conditions. The mulberry foliage is the sole source of nutrition for the mulberry silkworm (*Bombyx mori* L.). Foliar diseases pose a major bottleneck in production of quality mulberry leaves causing about 10-30% leaf yield loss. Besides significant quantitative loss, there is also reduction in the quality of leaves [1, 2]. *Myrothecium* leaf spot, bacterial leaf spot and powdery mildew are the prominent foliar diseases of mulberry in different seasons of the year in the Gangetic plains of West Bengal. *Myrothecium* leaf spot or brown leaf spot caused by *Paramyrothecium roridum* (Syn: *Myrothecium roridum* Tode ex. Fr.) is especially a major threat to mulberry cultivation in Eastern and North-Eastern India. The disease has been reported from India and Japan [3-5]. In India, the occurrence of the disease has been reported from Madhya Pradesh, Chhattishgarh, Assam, Bihar, Delhi, Gujarat, Karnataka, Kerala, Punjab, Rajasthan and Uttar Pradesh. The disease regularly appears during June and incidence lasts till November [6, 7]. With the occurrence of favorable weather conditions (30-32 °C temperature, 80 - 90% relative humidity and >10 rainy days/month), the disease causes 10 - 12% leaf yield loss by forming necrotic lesions followed by premature defoliation [8].

Cultural, mechanical, chemical and biological approaches either alone or in combination are employed to manage mulberry diseases. Though chemical fungicides are sprayed, often farmers are skeptical, since the leaves have to be fed to the silkworms. Improper use of agrochemicals could be dangerous to the silkworms and sprayed leaves can only be used after the mandatory safe period which is a great disadvantage. Constant use of chemicals also leads to the emergence of resistance, often decreasing the effectiveness. Moreover, they affect beneficial flora and fauna, pollute the environment and are harmful for the human health in the long run. These constraints warrant the search for effective replacements.

The use of biocontrol agents for plant disease management is a lucrative choice and has attracted substantial interest among the growers [9]. Biocontrol agents employ a variety of mechanisms viz., induced resistance, hyper parasitism, antibiosis, cross protection, competition for space and nutrient etc. against attacking pathogens [10]. With the ever increasing demand for biocontrol products among the farming community, future of biological control seems to be promising. With this view, the present study was attempted to isolate and identify bacterial antagonist(s) against brown leaf spot pathogen of mulberry.

Materials and methods

Location: The present study was carried out at Central Sericultural Research and Training Institute, Berhampore, West Bengal (Latitude - 24°5'28.01"N and Longitude 88°15'56.37"E).

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Isolation of pathogen causing brown leaf spot of mulberry: The pathogen was isolated from mulberry leaves showing typical symptoms of brown leaf spot. Leaf samples were washed under running tap water, cut into 1 cm² pieces, surface sterilized with 1% sodium hypochlorite solution for five minutes followed by thorough washing with autoclaved double distilled water, blot dried and were aseptically transferred on to 90mm Petri plates containing Potato Dextrose

Agar/PDA (Potato infusion-200g, Dextrose-20g, Agar agar-20g in 1000 ml Distilled water, pH- 5.6 ± 0.2). Petri plates were incubated at 25 °C and examined daily for the development of fungal growth. Pure cultures of the pathogen were obtained by repeated sub culturing on to PDA. Pure cultures were stored at 4 °C in refrigerator and intermittent transfer to fresh media was done every twenty one days.

Pathogenicity of the fungal isolate was confirmed by adopting Koch's postulates. The fungal strain was cultured on potato dextrose broth for 14 days. Healthy leaves of the mulberry plants maintained in the glasshouse were sprayed with spore suspension (106/ml) till run off. Inoculated plants were examined daily, for the development of symptoms. After confirming the pathogenicity, total genomic DNA was extracted and ITS region was amplified for molecular identification of the pathogen ^[11].

Isolation of bacterial biocontrol agents: Healthy mulberry leaves adjacent to infected mulberry leaves were collected from the mulberry gardens of CSR&TI, Berhampore. The leaves were packed in a sterile polythene bag to carry them back to the laboratory. For the isolation of bacteria, the method proposed by Vlassak *et al.* was adopted ^[12]. 10 g of leaf samples were taken in separate conical flasks to which 90 ml of distilled water was added (10-1). The sample was agitated for 15 minutes and serially diluted (10-2 to 10-6). 1 ml from 10-5 and 10-6 dilutions were added to sterilized Petri plates. 20 ml of molten and cooled nutrient agar (Peptone-5g, Beef extract-3g, Sodium chloride-5g, Agar agar-20g in 1000 ml distilled water, pH 6.8-7.2) was added to each plate and the plates were rotated clock wise and counter-clock wise. The Petri plates were incubated at room temperatures (28 °C ± 2 °C) for 24-72 hours. Two replications were maintained for each dilution. The plates were examined daily. Colonies were purified by repeated streaking onto fresh nutrient agar plates. Single colonies were sub-cultured onto fresh nutrient agar plates till identical colonies were observed on two successive plates. Contamination from other bacteria was checked by observing the characters of the culture like color of the culture, colony morphology and by examining microscopically.

Bacterial isolates with antagonistic activity were initially identified by dual culture technique ^[13]. Bacterial isolates were streaked onto nutrient agar and incubated for three days. Loopful of each bacterial isolate was streaked on to the PDA plate at one end, which was pre- inoculated with 14 days old, 5 mm mycelial disc of the pathogen at the other end. Control plate was maintained by placing only mycelial disc of the pathogen on the plate without streaking the bacterium. The inoculated plates were incubated at 25 ± 2 °C until the growth in control plate, covered the Petri dish. Promising isolates were selected for further evaluation.

Evaluation of antifungal activity of bacterial antagonists: The antagonistic activity of selected bacterial isolates against brown leaf spot pathogen was further evaluated by dual

culture technique ^[13]. Promising bacterial isolates along with a fungal biocontrol agent *Trichoderma* sp. (for comparison) were evaluated for their ability to suppress the radial growth of the pathogen. Control plates were maintained by placing only mycelial disc of the pathogen on the plate without bacteria. Completely Randomized Design (CRD) with three replications for each treatment was followed. The inoculated plates were incubated at 25 ± 2°C. The effect of bacteria on plant pathogen was determined by calculating Percent growth Inhibition (PI). The PI over control was calculated using the formula,

Gram's staining

Gram-staining of the bacterial isolate under test was performed using a Gram-staining kit [HiMedia (Cat No: K001)] following manufacturer's instructions. The stained bacterial cells were visualized under phase contrast microscope at 100 X magnification.

Molecular identification: 2-3 well grown isolated colonies were picked up from the plate and suspended in 50 µl of nuclease free water. The bacterial suspension was lysed in a thermal cycler (Eppendorf Nexus gradient mastercycler) at 92 °C for 12 min. The lysate was used as template for PCR amplification of 16S rRNA gene. Amplification was performed using thermal cycler (Eppendorf Nexus gradient mastercycler) in 25µl volumes taken in 0.2 ml microfuge tubes. The universal primers used for the amplification of the 16S rRNA gene were Eub27F (5'GAGTTTGATCCTGGCTCAG 3') and Univ1492R (5'GGTTACCTTGTTACGACTT 3'). The PCR reaction mixture was prepared by adding 1 µl of each primer (forward and reverse), 2.5 µl template DNA, 8 µl DNase free water and 12.5 µl master mix (Himedia). The mixture was subjected to one cycle of initial denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 40 sec, annealing at 50 °C for 40 sec, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Upon completion of the reaction, the products were stored at 4 °C until further use.

Analysis of PCR products by agarose gel electrophoresis:

Amplification of the 16S rRNA gene by polymerase chain reaction was confirmed by performing agarose gel electrophoresis [2% (w/v) in TAE buffer at 15 V cm⁻¹] of 5 µl of amplicon and 5 µl DNA marker (O'Gene Ruler™ 1 kb DNA Ladder). The gel was stained in Ethidium bromide solution (0.5 µg ml⁻¹) and visualized in a gel documentation system (Gel Doc EZ imager of Biorad).

Sequencing: Upon confirmation of the amplification, the 16S rRNA gene amplicon was sequenced at M/s. Xcelris Labs, Gujarat, India (Sanger sequencing on ABI 3730xl 96 capillary system using BigDye Terminator v3.1 kit).

BLAST search and Phylogenetic analysis: Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were done using the EzBioCloud server (<http://www.ezbiocloud.net/eztaxon>). The sequence was also alternatively submitted to NCBI-BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis. Based on the BLAST search results from the EzBioCloud server, sequences of the closely related type strains and an out group were retrieved in FASTA format from National Center for Biotechnology Information (NCBI-<http://www.ncbi.nlm.nih.gov/nucleo>). The type strain

accession numbers were obtained from List of prokaryotic names with standing in nomenclature (LPSN-<http://www.bacterio.cict.fr/index.html>). The CLUSTALW algorithm of MEGA 6.0 was used for sequence alignments and MEGA 6.0 software was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner. Neighbour-joining (NJ) method in the MEGA 6.0 software was used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure based on 1000 replications.

Results and Discussions

The present study was aimed at isolating and characterizing phylloplane bacteria that are effective in suppressing pathogen causing brown leaf spot of mulberry and the results are presented below.

Isolation of the pathogen: Brown leaf spot pathogen was isolated from mulberry leaves of variety S1635, exhibiting typical symptoms *viz.*, brown necrotic spots that were dark brown to black in color surrounded by yellow hallow. These spots were round to irregular in shape and found anywhere on the leaves. With the progress of time, smaller spots coalesced to form blighted areas. In advanced stages, severely infected leaves turned yellowish leading to premature defoliation. Irregularly shaped, raised, black sporodochia with a white fringe of mycelium in concentric rings, within the necrotic areas were seen on the lower side of the leaves.

On PDA, the pathogen developed white flucrose colonies with sporodochia in dark green to black concentric rings bearing viscid masses of conidia. Conidia were hyaline to slightly dark, one-celled, ovoid to elongate with rounded ends while conidiophores were subhyaline to green cloured, repeatedly branched, bearing conidia terminally typical of the pathogen. Pathogenicity was confirmed by adopting Koch's postulates. Within two weeks of spraying the potted plants with the spore suspension, the fungal infection was observed on the leaves of the plant. Identity of the pathogen was confirmed by sequencing the ITS region from the DNA of the causal agent which was amplified using primers ITS1/ITS4 [11].

Isolation of bacterial antagonists: Bacteria were isolated from mulberry phylloplane following serial dilution and plating. In total, fifty bacterial isolates were purified by streak plate method. Bacterial isolates with antagonistic activity was initially identified by dual culture technique. Out of fifty, five bacterial isolates showed inhibition of mycelial growth (visible presence of clear zone of inhibition) of the pathogen and were designated as MP-18-1 to MP-18-5 (Mulberry Phylloplane, 2018). The selected isolates were maintained on nutrient agar slants by periodic sub-culturing.

Evaluation of antifungal activity of bacterial antagonists: Five promising bacterial isolates exhibiting antifungal activities identified from preliminary screening were further evaluated, along with the fungal biocontrol agent *Trichoderma* sp. All the five bacterial isolates (MP-18-1 to MP-18-5) were found to have antifungal activity against *Paramyothecium roridum* but differed in their potential as

indicated by the diameter of zones of inhibition while *Paramyothecium roridum* inoculated in the control plates exhibited luxuriant growth all along the Petri dish (Fig. 1A). The diameter of the culture growth of the fungal pathogen in each of the plates was measured and compared with that of the control (Fig. 1B). All the isolates significantly reduced the radial growth of the pathogen (Table 1), however fungal biocontrol agent *Trichoderma* sp. was most effective in suppressing the mycelial growth of the pathogen with 74.9% inhibition. Among the bacterial isolates, MP-18-5 was most effective and showed 62.0% mycelial growth inhibition. Bacterial isolates MP-18-4 and MP-18-1 were at par with each other and exhibited 52.3% and 51.1% radial growth inhibition, respectively. Similarly MP-18-2 and MP-18-3 also reduced the mycelial growth of the pathogen by 35.6% and 35.2% and were found to be at par with each other.

Table 1: Effect of biocontrol agents on the radial mycelial growth of *Paramyothecium roridum* under in vitro conditions

Treatment*	Percent growth inhibition (%)**
T1: MP-18-1	51.1 (7.1#)c##
T2: MP-18-2	35.6 (6.0)d
T3: MP-18-3	35.2 (5.9)d
T4: MP-18-4	52.3 (7.2)c
T5: MP-18-5	62.0 (7.9)b
T6: <i>Trichoderma</i> sp.	74.9 (8.7)a
SEm±	0.15
CV	3.69
CD (5%)	0.46
CD (1%)	0.67

*Inhibition in control plates was taken as zero

**Mean of three replications

#Figures in the parenthesis are square root transformed values

##Means with same letter are not significantly different

Gram staining: Most effective bacterial isolate MP-18-5 was found to be Gram positive and rod shaped.

Molecular identification: Total genomic DNA was extracted from bacterial isolate MP-18-5 and PCR amplification of 16S rRNA region from the same was confirmed by agarose gel electrophoresis (Fig. 2). Based on EZBioCloud search analysis which compares the 16S rRNA gene sequences with type strain sequences, the bacterial antagonist MP-18-5 was found to share 97.79% similarity with the type strains of *Bacillus zhangzhouensis* (JOTPO1000061), *B. safensis* subsp. *safensis* (ASJD01000027), *B. safensis* subsp. *osmophilus* (KY990920). It also shared 97.61% similarity with the type strains of *B. pumilus* (ABRX01000007) and *B. australimaris* (JX680098). The results from the EZBioCloud search analysis are in agreement with the phylogenetic tree (Fig. 3) that was constructed with the 16S rRNA gene sequences of the isolate from the present investigation and sequences of its closest phylogenetic neighbours downloaded from the NCBI GenBank. NCBI BLAST search analysis revealed that sequence of the bacterial antagonist had 96.74% similarity with *B. zhangzhouensis* LA361a (KY622435), *B. safensis* CAIM 388 (HM583998) and *B. pumilus* BPK-5 (GQ352639). It also shared a 96.58% nucleotide similarity with *B. safensis* DMB33 (KT274778) and strain 003157 (HE970652) from India.

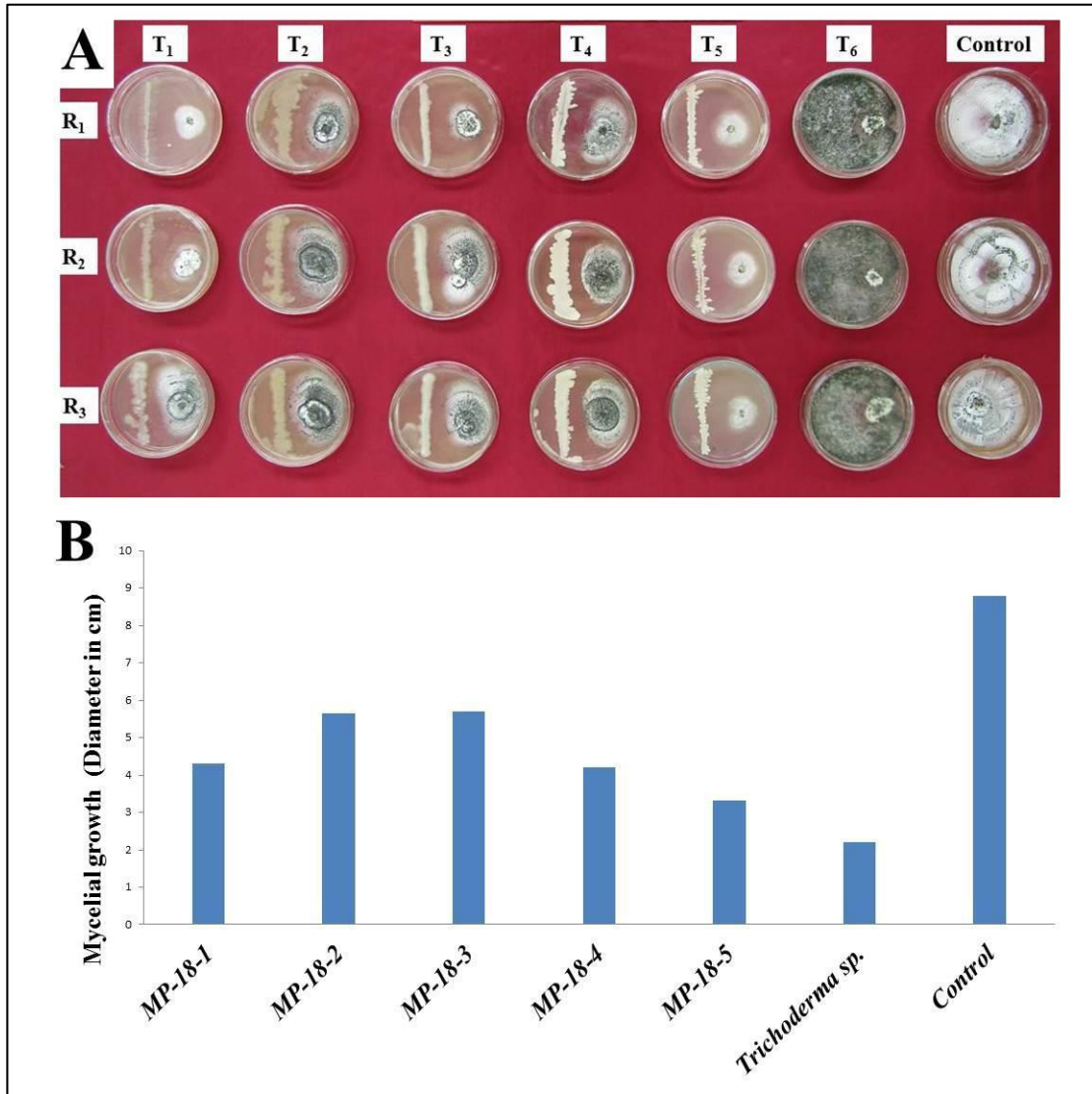


Fig 1: Effect of biocontrol agents on the radial mycelial growth of *Paramyrothecium roridum*

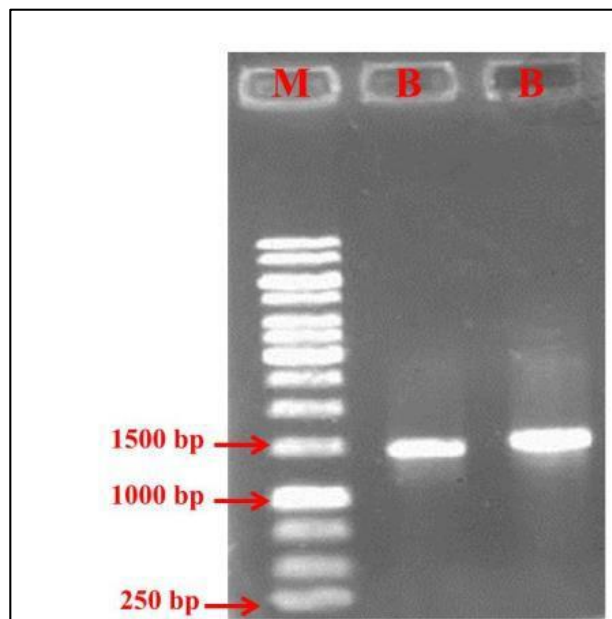


Fig 2: Ethidium bromide stained agarose gel depicting amplified 16S rRNA gene of bacterial isolate MP-18-5 (M: 1 Kb DNA ladder, B: PCR amplicon of 16S rRNA gene of bacterial isolate MP-18-5)

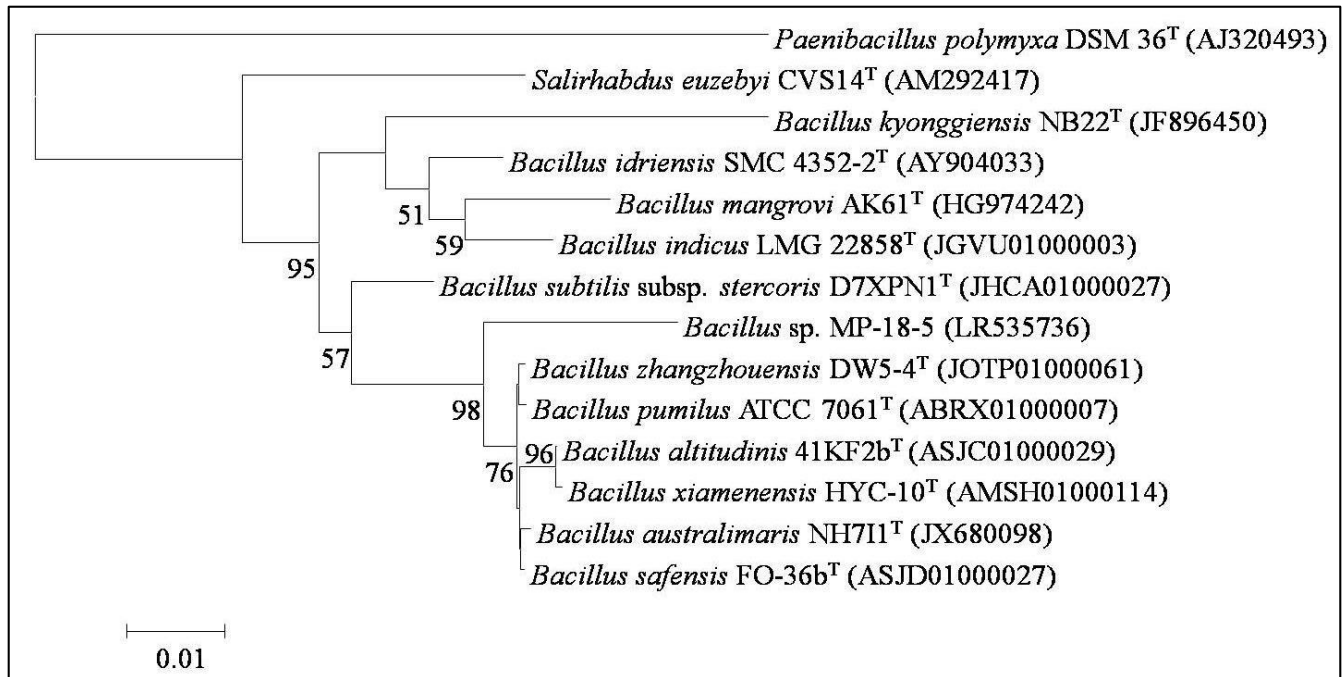


Fig 3: NJ tree based on 16S rRNA gene sequences showing phylogenetic relationship of the bacterial strain MP-18-5 isolated in the present study with its closest phylogenetic neighbours. Numbers at nodes represent bootstrap percentages. GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

There are ample reports indicating the usefulness and effectiveness of *Bacillus* spp. in biocontrol of plant diseases. Recently Karagoz *et al.* identified *Bacillus zhangzhouensis* by 16S rRNA gene sequencing and the isolate has been found to be an effective biocontrol agent against common scab disease caused by *Streptomyces scabiei* [14]. Similarly, application of *B. pumilus* reduced incidence of *Fusarium* wilt disease of tomato by 73% and significantly increased root and shoot length [15]. *B. pumilus* suppressed *Sclerotinia* stalk rot disease severity by 93% and increased yield of cauliflower by 36% in Himachal Pradesh [16]. Biocontrol potential of *B. pumilus* is also evident from the works of Akhtar *et al.* and Ren *et al.* who reported the effectiveness of *B. pumilus* against wilt disease of lentil caused by *Fusarium oxysporum* f. sp. *lentis* and canker disease of poplar respectively [17, 18].

Many prospective biocontrol agents belong to Genus *Bacillus* because of their numerous desirable characters [19]. Antimicrobial activities of *Bacilli* are mainly attributed to the production of antibiotics (Iturin, Mycosubtilin etc.) and siderophores [20]. *Bacillus amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. pumilus* and *B. subtilis* are known to produce many antifungal and antimicrobial compounds [20, 21]. It is estimated that 4-5% of the genome of *B. subtilis* is devoted to the antibiotic production and it is reported to produce a number of structurally diverse antimicrobial compounds [22]. For instance, Manjula and Podile reported that *B. subtilis* AF1 produced N-acetyl glucosaminidase and glucanase which are toxic to fungi [23]. The members of the genus *Bacillus* are also found to elicit growth and defense responses in the plants [24]. In addition, *Bacilli* form endospores that are extremely resistant to high temperature, adverse pH, lack of nutrients or water, etc. [25]. This is a great advantage as bacterial cultures can be transformed into easy to handle formulations without losing viability, one of the problems while working with bacteria that do not sporulate [26, 27]. Moreover bacteria such as *B. subtilis*, are widely studied, which also facilitates their effective exploitation in biocontrol.

Formulations based on *Bacillus pumilus* viz., Ballad and Sonata (Agra Quest Inc., USA), Yield Shield (Bayer Crop Science, USA) are already available in the market which exemplifies the commercial value of *Bacilli* as biocontrol agent. According to Hélène *et al.* about half of the commercially available bacterial biocontrol agents belong to genus *Bacillus* [28]. Considering the versatility of the genus *Bacillus*, it was not a surprise to identify *Bacillus* sp. with antifungal activities against *Paramyothecium roridum* by dual culture method in the present investigation. Detailed studies have to be carried to elucidate the source of antifungal activity of the identified bacterium in the present study. Studies also need to be performed to assess the potential of the same for utilization in the field.

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