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Endophytic Bacillus spp. from medicinal plants inhibit mycelial growth of Sclerotinia sclerotiorum and promote plant growth

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Endophytic *Bacillus* spp. from medicinal plants inhibit mycelial growth of *Sclerotinia sclerotiorum* and promote plant growth

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Abstract: Plant growth-promoting bacteria that are also capable of suppressing plant pathogenic fungi play an important role in sustainable agriculture. There is a critical need for conducting research to discover, characterize and evaluate the efficacy of new strains of such bacteria in controlling highly aggressive plant pathogens. In this study, we isolated endophytic bacteria from medicinal plants of Bangladesh and evaluated their antagonistic capacity against an important phytopathogenic fungus *Sclerotinia sclerotiorum*. Growth-promoting effects of those isolates on cucumber and rice seedlings were also assessed. Among 16 morphologically distinct isolates, BDR-2, BRtL-2 and BCL-1 significantly inhibited the growth of *S. sclerotiorum* through induction of characteristic morphological alterations in hyphae and reduction of mycelial dry weight. When cucumber and rice seeds were treated with these endophytic bacteria, seven isolates (BCL-1, BDL-1, BRtL-2, BRtL-3, BDR-1, BDR-2 and BBoS-1) enhanced seed germination, seedling vigor, seedling growth and number of roots per plant at a varying level compared to untreated controls. All isolates produced high levels of indole-3-acetic acid (6 to 63 μg/mL) in vitro. Two most potential isolates, BDR-2 and BRtL-2, were identified as *Bacillus amyloliquefaciens* and *B. subtilis*, respectively, based on the 16S rRNA gene sequencing. These results suggest that endophytic *Bacillus* species from native medicinal plants have great potential for being used as natural plant growth promoter and biopesticides in sustainable crop production.

Keywords: biological control; endophytic bacteria; growth promoter; *Sclerotinia sclerotiorum*.

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1 Introduction

Endophytic bacteria are ubiquitous microorganisms that live within a living plant without causing any apparent harm to the host plant [1]. Search for beneficial endophytic microorganisms from traditional medicinal plants and their application in sustainable agricultural practices has been increasing all over the world [2, 3]. *Bacillus, Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, *Paraburkholderia* and *Serratia* have already been reported as plant growth-promoting rhizobacteria and biocontrol agents [3–8].

Among various reported plant growth-promoting and biocontrol bacteria, species of *Bacillus* showed the highest potential both in vitro and in vivo [9]. For example, *B. subtilis* produces various phytohormones such as indole-3-acetic acid (IAA), cytokinins, zeatin, gibberellic acid and abscisic acid that are transported into the shoot through the xylem, delay senescence and thus boost production of lettuce, tomato, cucumber and pepper [10, 11]. Apart from the improvement of crop yield, *B. subtilis* also induces resistance to the fungal phytopathogens. Strains of *B. amyloliquefaciens* and *B. subtilis* are known to competitively colonize plants and can simultaneously act as biofertilizer and antagonists (biopesticides) of bacteria, fungi,

peronosporomycetes and nematodes [12–14]. Colonization of cucumber plants by *B. polymyxa* increased enzymatic activities with a concurrent increase in cucumber yield up to 25% compared to non-treated control [15]. Production of growth-stimulating phytohormones, solubilization and mobilization of phosphates, production of siderophores, production of antibiotics, inhibition of plant ethylene synthesis and induction of plant systemic resistance to pathogens are considered as the mechanisms of plant growth promotion by endophytic bacteria [2, 3, 13, 16, 17].

Sclerotinia sclerotiorum is the most destructive phytopathogen with a wide host range, which has been reported to infect 64 plant families [18, 19]. It causes white mold, cottony rot, watery soft rot, stem rot, drop, crown rot and twig blight and many other serious diseases in plants resulting in significant yield losses worldwide [18]. In the field condition, yield loss may exceed 50% due to *Sclerotinia* blight in peanut under a favorable environmental condition. Control of plant diseases caused by *S. sclerotiorum* by synthetic fungicides is problematic due to high cost, low efficacy and deleterious effects on the environment [19]. Biological agents can be the best alternatives for controlling plant diseases due to the reasons mentioned above [5, 7, 20]. Bangladesh is rich in diversity of medicinal plants from which discovery of novel endophytic bacteria to use as the biopesticides against notorious phytopathogens including *S. sclerotiorum* holds great potential. No systematic study has so far been conducted on isolation and identification of endophytic bacteria from medicinal plants of Bangladesh, and has evaluated their effects on growth promotion and protection of plants from phytopathogens. Therefore, the objectives of this study were to (i) isolate and identify potential endophytic bacteria from some important medicinal plants; (ii) evaluate inhibitory effects of the isolated endophytic bacteria against *S. sclerotiorum* and (iii) assess the effects of endophytes on seed germination, seedling vigor and growth of rice and cucumber.

2 Materials and methods

2.1 General experimental procedure

All the chemicals and reagents used in this study were available at the Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh, previously procured from Merck AG, Germany. All photographs were taken by a high-resolution camera attached to a compound microscope.

2.2 Source and preservation of *Sclerotinia sclerotiorum* strain

The pathogen *S. sclerotiorum* was collected from the stock culture of the Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh, which was previously isolated from hyacinth bean [21]. This organism was regularly cultured on the Potato Dextrose Agar (PDA) medium and preserved in a PDA slant.

2.3 Isolation of endophytic bacteria

Sixteen endophytic bacteria were isolated from different well-known medicinal plant parts such as root and leaf of *Duranta plumeri*, leaf of *Ocimum gratissimum* L., seed of *Terminalia bohera* and leaf of *Manihot esculenta* from Dhaka, Gazipur and Rajshahi districts of Bangladesh. Five-gram fresh plant sample from each of different organs were washed under running tap water and surface sterilized with 70% ethanol for 10 min followed by 1% NaOCl for 1 min and finally washed with sterile distilled water (SDW) three times to remove excess NaOCl [2]. Tissue pieces were then smashed in a sterilized mortar and pestle. Smashed plant materials were serially diluted up to 1×10^{-6} in SDW, from which 100 µL was spread evenly on petri dishes containing yeast extract glucose agar (YGA) medium and incubated at 25 °C for 24 h. Bacterial colonies were isolated based on the colony color, shape and another unique identification criterion, and purified by repeated streak plate culture. The purified single colony isolates were preserved in 20% glycerol solution at –20 °C for subsequent experiments.

2.4 Morphological and biochemical characterization of endophytic bacterial isolates

Morphological characteristics of colonies such as size, shape, color and growth pattern were recorded under a light microscope after 24 h of growth on the YGA medium at 25 ± 2 °C as described earlier [22]. A series of biochemical tests were performed to characterize the isolated endophytic bacteria using the protocols described in Bergey's Manual of Systematic Bacteriology [23]. For the KOH solubility test, endophytic bacteria were mixed aseptically with 3% KOH solution on a clean slide with an inoculating wire loop for 1 min and observed for the formation of

a thread-like mass. Catalase and oxidase tests were performed as described earlier by Hayward [24] and Rajat et al. [25], respectively.

2.5 Molecular identification of isolated endophytic bacteria

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed followed by sequencing to identify the active bacterial strains [7]. Initially, cells from the single bacterial colony were harvested from agar broth and re-suspended in 100 µL SDW by vortexing for 10 s. For the determination of 16S rRNA sequences of active strains, chromosomal DNA extraction was done using a commercial DNA extraction kit (ATP Biotech Inc., Taiwan) and quantified comparing with the lambda DNA marker after agarose gel electrophoresis. The 16S rRNA region was amplified by PCR using a universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was carried out in Mastercycler® Gradient (Eppendorf, Hamburg, Germany) for 30 successive cycles consisting of 5 min of initial denaturation at 94 °C, 40 s of denaturation at 94 °C, 40 s of annealing at 55 °C and 1 min of extension at 72 °C with a final extension for 10 min at 72 °C. PCR mix was purified and analyzed followed by sequencing with Big Dye terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequences were combined using the Laser gene version 7.1 program. The 16S rRNA gene sequences of the strains were then subjected to BLAST search using the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for DNA-DNA homology.

2.6 Determination of *S. sclerotiorum* growth suppression by endophytes

To assess antagonistic activities, seven bacterial strains were tested against *S. sclerotiorum* using a dual culture assay. A 6-mm-diameter mycelial plug of the pathogen was cut from an actively growing colony with a sterile cork borer and placed 3 cm apart from each endophyte colony on the PDA plate [26]. The inhibition zone was observed after incubation at 25 °C for 72 h. The radial growth of the pathogen and percentage inhibition was calculated after 10 days as described by Zohara et al. [7].

$$\% \text{ Inhibition of growth} = \frac{X - Y}{X} \times 100$$

where X is the mycelial growth of the pathogen in the absence of the antagonist, and Y is the = mycelial growth of the pathogen in the presence of the antagonist.

Morphological characters of approaching hyphae were observed under a light microscope and images were recorded with a digital camera attached with the microscope. At least five samples were examined for each combination of the pathogen and antagonistic endophyte.

2.7 Effect of endophytic bacterial strains on mycelial dry weight of *S. sclerotiorum*

Quantitative antagonistic effect of isolated endophytic bacterial strains was measured. Briefly, a block of *S. sclerotiorum* was grown in a conical flask containing 200 mL potato dextrose broth (PDB) for 3 days. After 3 days, 1 mL of 1×10^{5}-, 1×10^{6}-, 1×10^{7}- and 1×10^{8}-fold diluted bacterial cells were added to the broth and incubated at 25 °C for 10 days. Before inoculation of bacterial strains, mycelial dry weight of control flasks was measured to determine whether bacterial strains can decompose and decrease mycelial growth of *S. sclerotiorum*. After 10 days of pathogen mycelia and bacteria interaction, mycelia from each flask were placed on separate filter paper and allowed to dry for 3 h, after which time mycelial dry weight was recorded individually to determine the effect of each antagonistic bacterial strain.

2.8 Plant growth promoting activity of the endophytic bacteria

2.8.1 Indole-3-acetic acid production

For determination and quantification of IAA production, colonies of bacterial strains were inoculated into Jensen's broth (Sucrose 20 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $FeSO_4$ 0.1 g/L[1], $NaMoO_4$ 0.005 g/L, $CaCO_3$ 2 g/L) [27] containing 2 mg/mL L-tryptophan and incubated at 28 ± 2 °C with continuous shaking at 125 rpm for 48 h on a benchtop shaker. Two milliliters of culture solution was centrifuged at 12,000 *g* for 1 min, and 1 mL aliquot of the supernatant was mixed with 2 mL of Salkowski's reagent (150 mL conc. H_2SO_4, 250 mL distilled water, 7.5 mL 0.5 M $FeCl_3 \cdot 6H_2O$) and incubated for 20 min in darkness at room temperature, as described by Gordon and Weber [28]. IAA production was indicated by the development of a pink-red color, and the absorbance

was measured at 530 nm using a spectrophotometer (PD-303U, APEL, Japan). The concentration of IAA was determined using a standard curve prepared from pure IAA solutions (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 µg/mL). Supernatants from un-inoculated test tubes were used as control.

2.8.2 Preparation of bacterial inocula for seed treatment

Bacterial strains that were isolated from medicinal plants were cultured separately in conical flasks containing 200 mL yeast peptone broth and incubated in an orbital shaker at 120 rpm for 72 h at 25 °C for preparation of inocula. Bacterial cells were collected via centrifugation at 15,000 rpm for 1 min at 4 °C. After washing with SDW, bacterial pellets were suspended in 0.6 mL SDW, vortexed and used for seed treatment. One gram of cucumber and rice seeds were surface sterilized with 70% ethanol for 10 min followed by washing with SDW. Seeds were then soaked in bacterial suspension in a petri dish (for how long?) and dried overnight at room temperature to ensure better coating with bacteria. The number of bacterial cells per seed was ca. 1×10^8 CFU, counted by using the serial dilution technique. Inoculated seeds were planted in pots containing soil mix. After germination, seedlings were watered on alternate days.

2.8.3 Effect of bacterial seed treatment on germination and vigor index of cucumber and rice

For determination of percent germination and seedling vigor, 100 seeds inoculated with each strain were incubated at 25 °C in petri dishes (9 cm) in three replications on a layer of moistened filter paper. Seeds soaked in water instead of bacterial suspensions were used as control. Water was added to the petri dishes to maintain sufficient moisture. The germination percentage was recorded every 24 h for 7 days. Root and shoot lengths were measured after the seventh day.

The germination percentage and vigor index were calculated using the following formula:

$$\text{Germination (\%)} = (\text{No. of seeds germinated} / \text{Total no. of seeds sown}) \times 100$$

$$\text{Vigor index} = [\text{Mean shoot length (cm)} + \text{Mean root length (cm)}] \times \text{Germination \%}.$$

2.9 Statistical analysis

Data from different experiments were analyzed using the statistical software SPSS (Version 15) and the Microsoft Office Excel 2007 program and XL STAT (Version. 2012). Fisher's protected LSD test was used to determine the levels of significant differences among the mean values at $p \leq 0.05$. The experimental design was completely randomized, consisting of at least three replications for each treatment and were repeated at least twice.

3 Results

3.1 Isolation, screening and identification of endophytic bacteria

A total of 16 endophytic bacteria were isolated and purified from medicinal plants by repeated streak plate culture on the YGA medium. Initially, all strains were screened against *S. sclerotiorum* in a dual culture agar plate for assessing their antagonistic activities. Three bacterial strains, namely BCL-1, BRtL-2 and BDR-2, out of 16 showed vigorous but varying level of hyphal growth inhibition against *S. sclerotiorum*. Ribosomal gene sequences (16S rRNA) of two most potent antagonists were done for identification and deposited in the GenBank. The 16S rRNA sequences of BRtL-2 and BDR-2 showed 100% similarity with *B. subtilis* strain Tc1 (accession number GU391355.1) and *B. amyloliquefaciens* strain D41 (accession number KC441776.1), respectively. The isolates BRtL-2 and BDR-2 were isolated from two famous traditional medicinal plants, namely, *O. gratissimum* L. (leaf) and *D. plumeri* (root).

3.2 Biochemical characterization of endophytic bacteria

Based on in vitro antagonism against *S. sclerotiorum* and ability to produce IAA, seven isolates, namely, BDR-1 (root of *D. plumeri*), BDR-2 (root of *D. plumeri*), BDL-1 (leaf of *D. plumeri*), BRtL-2 (leaf of *O. gratissimum* L.), BRtL-3 (leaf of *O. gratissimum* L.), BBoS-1 (seed of *T. bohera*) and BCL-1 (leaf of *M. esculenta*), were selected for further morphological and biochemical study. All isolates were brown to whitish in color, fast-growing on various culture media and produced colonies of round to irregular shape with smooth surfaces. All seven isolates reacted positively to the catalase test and Gram-staining test except BRtL-3.

Only four isolates (BDL-1, BDR-1, BDR-2 and BRtL-2) reacted positively to the oxidase test. All but BRtL-3 reacted negatively to the KOH solubility test.

3.3 Indole-3-acetic acid production by endophytic bacteria

Production of IAA by the isolates was examined as it is an important trait of plant growth promotion by the endophytic bacteria. In the presence of tryptophan, isolated endophytic bacteria produced IAA in concentrations between 6.3 and 63 µg/mL. The highest IAA was produced by isolate BRtL-3 (63 µg/mL) followed by BDL-1 (60.8 µg/mL), *B. amyloliquefaciens* BDR-2 (58.6 µg/mL) and BBoS-1 (52.3 µg/mL). However, the lowest IAA production (6.3 µg/mL) was found in *B. subtilis* BRtL-2. IAAs produced by BDR-1 and BCL-1 were 8.6 and 12.6 µg/mL, respectively. These results suggest that isolated endophytes remarkably varied in IAA production, and thus opportunity does exist to further explore for higher IAA-producing isolates. However, these results also suggest that endophytes with higher IAA production capacity may not show higher antagonistic activity against phytopathogens. Thus, screening of endophytes should consider all relevant criteria for selecting isolates.

3.4 In vitro interactions between *Bacillus* spp. and *S. sclerotiorum*

In a dual culture assay, hyphal growth of *S. sclerotiorum* toward the colonies of *B. amyloliquifaciens* BDR-2, *B. subtilis* BRtL-2 and BCL-1 was remarkably inhibited compared with the untreated control plate (Figure 1A–D). Clear inhibition zones formed on the agar medium due to the interaction between antagonistic bacteria and *S. sclerotiorum*. The largest inhibition of radial growth was recorded in BCL-1 (69.1%) followed by *B. amyloliquefaciens* BDR-2 (66.7%) and *B. subtilis* BRtL-2 (54.7%). The percentages of hyphal growth inhibition by the bacterial endophytes were statistically different from each other as well as the untreated control plates (Table 1). No sclerotia were produced in plates treated with bacterial endophytes, whereas sclerotia were plentiful in the control plates.

Distinct morphological alterations such as irregular and excessive branching, abnormal swelling of hyphal diameter, unusually long and pointed hyphal tips and hyphal lysis of *S. sclerotiorum* were observed microscopically in approaching hyphae toward the colonies of bacterial endophytes (BCL-1, BRtL-2 and BDR-2) (Figure 1B–D). Normal polar growth of hyphae was observed under a microscope in the untreated control plates (Figure 1A). Interactions with *B. subtilis* BRtL-2 caused disruption of normal radial growth of hyphae by inducing excessive branching, curling, swelling and pointed hyphae (Figure 1B). Stunted mycelial growth with swelling, pointed tips and excessive branching was observed in *S. sclerotiorum* due to interaction with endophytic bacterial strain BCL-1 (Figure 1C). The interaction of *S. sclerotiorum* with *B. amyloliquifaciens* BDR-2 resulted in hyper-branching, curly growth and broke down of hyphae (Figure 1D). Although excessive branching and loss of polar growth in *S. sclerotiorum* were common, each strain in addition induced characteristic morphological alterations, indicating production of diverse inhibitory compounds by the bacterial antagonists.

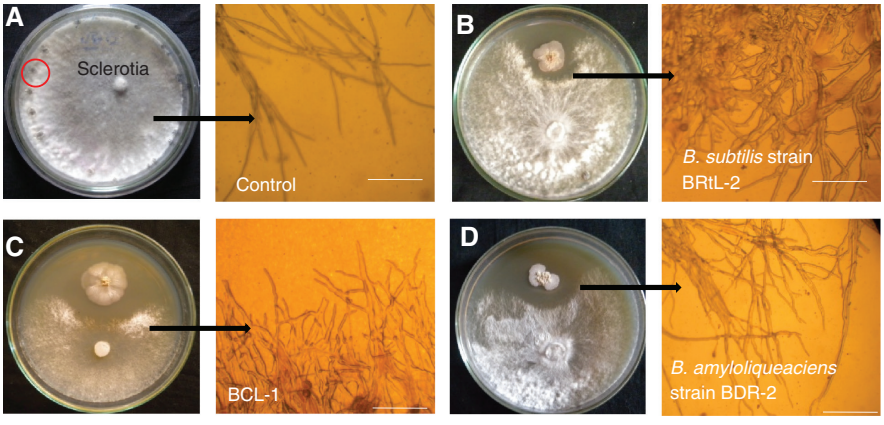

Figure 1: Interactions between antagonistic *Bacillus* spp. and *Sclerotinia sclerotiorum* in dual culture assay in PDA and photo microscopic view of morphological alterations in *S. sclerotiorum* hyphae (A–D). (A) Normal mycelia with sclerotia (round red mark) of *S. sclerotiorum* in untreated control; (B) excessive branching with curling by *Bacillus subtilis* strain BRtL-2; (C) excessive branching, pointed tips and cellular disintegration by BCL-1 and (D) cellular disintegration and swelling of *S. sclerotiorum* hyphae by *Bacillus amyloliquefaciens* strain BDR-2. Scale bars = 100 µm.

Table 1: Effects of *Bacillus* spp. on in vitro reduction of mycelial growth and dry weight of *Sclerotinia sclerotiorum*.

Bacterial strains	Pathogen suppression[a]			Reduction of mycelial dry weight (g)[b]	
	Radial growth of *S. sclerotiorum* (cm)	Sclerotial number	Percentage of mycelial growth inhibition of *S. sclerotiorum*	Mycelial dry weight after 10 days	Percentage of dry weight reduction
BCL-1	1.4 ± 0.04a	0 ± 0	69.1 ± 0.3a	2.4 ± 0.03	26.9
Bacillus subtilis strain BRtL-2	2.0 ± 0.03b	0 ± 0	54.7 ± 0.6b	1.8 ± 0.01	46.3
Bacillus amyloliquifaciens strain BDR-2	1.5 ± 0.08a	0 ± 0	66.7 ± 0.7a	1.8 ± 0.01	47.3
Control	4.5 ± 0.01c	25.7 ± 1.2	0.0 ± 0.0c	3.3 ± 0.01	0.00

Mean values within the same column followed by different letters are significantly different by Duncan's Multiple Range Test (DMRT) at p = 0.05. Data presented here are mean value ± SE of at least three replications. [a]In vitro suppression of mycelial growth and sclerotia production of *S. sclerotiorum*. [b]Mycelial dry weight of *S. sclerotiorum* was measured after adding bacterial inocula in conical flasks and incubation for 10 days.

3.5 Reduction of mycelial dry weight of *S. sclerotiorum* by the endophytic bacteria

Mycelial growth of *S. sclerotiorum* was remarkably affected by three bacterial antagonists in the PDB medium when bacterial isolates were added to the conical flasks containing 3 days' old culture of *S. sclerotiorum* (Table 1). After 10 days of incubation, the highest *S. sclerotiorum* mycelial dry weight (3.3 ± 0.01 g) was found in non-inoculated control flasks among all the treatments. The mycelial dry weight of *S. sclerotiorum* was remarkably decreased in the presence of *Bacillus* spp. The lowest mycelial dry weight (1.8 g) was found in a *B. amyloliquifaciens* BDR-2-treated flask followed by 1.8 and 2.4 g in *B. subtilis* BRtL-2- and BCL-1-treated flasks, respectively (Table 1).

3.6 Effect of endophytic bacteria on seed germination and vigor index of *Cucumis sativus*

All treatments improved seed germination and vigor index of cucumber seedlings compared to untreated control. Hundred percent germination of cucumber seeds was obtained in all bacterial isolates used in seed treatment except BBoS-1-treated seeds (98.3 ± 2.8%). Lowest germination percentage (96.7 ± 2.8%) was recorded in untreated control (Table 2). Seedlings obtained from endophyte-treated seeds showed an enhanced plant vigor index compared to untreated control. Treatment with BRtL-3 showed the highest vigor index of (871.6 ± 40), which was statistically similar to other treatments except BDR-1 (515.7 ± 25) but far higher than the untreated control (314.8 ± 30) (Table 2).

Table 2: Effect of endophytic bacteria on seed germination, seedling vigor, growth and dry matter production of cucumber seedlings grown in vitro under axenic conditions.

Bacterial strains	Germination (%)	Vigor index	Shoot length (cm)	Root length (cm)	Shoot fresh weight (mg)	Shoot dry weight (mg)	Root fresh weight (mg)	Root dry weight (mg)
BCL-1	100 ± 0	966.67 ± 23	4.3 ± 0.3bc	5.3 ± 0.5a	116.6 ± 1.2c	18.3 ± 0.8ab	25.0 ± 3.0d	0.3 ± 0.1a
BDL-1	100 ± 0	733.33 ± 25	3.8 ± 0.2c	3.5 ± 0.3b	113.2 ± 1.7c	17.9 ± 1.0ab	32.5 ± 1.7bc	0.2 ± 0.1ab
BRtL-2	100 ± 0	803.33 ± 30	4.7 ± 0.2ab	3.3 ± 0.4bc	114.1 ± 0.9c	15.0 ± 0.8cd	23.2 ± 2.3d	0.1 ± 0.1b
BRtL-3	100 ± 0	790 ± 40	3.1 ± 0.2d	4.9 ± 0.3a	131.3 ± 0.6b	19.8 ± 0.7a	35.7 ± 1.1ab	0.3 ± 0.1a
BDR-1	100 ± 0	533.33 ± 25	2.2 ± 0.2e	3.2 ± 0.6bc	86.0 ± 2.6e	14.3 ± 1.2d	17.8 ± 0.9e	0.2 ± 0.1ab
BDR-2	100 ± 0	665 ± 30	3.0 ± 0.0d	3.8 ± 0.2b	108.1 ± 2.4d	17.4 ± 1.0abc	36.5 ± 1.7a	0.2 ± 0.1ab
BBoS1	98.33 ± 2.8	862.02 ± 23	5.2 ± 0.2a	3.6 ± 0.3b	159.1 ± 1.9a	16.7 ± 0.9bcd	29.3 ± 1.5c	0.2 ± 0.1ab
Control	96.7 ± 2.8	306.22 ± 30	0.5 ± 0.0f	2.7 ± 0.8c	33.2 ± 0.6f	8.4 ± 0.5e	16.5 ± 2.5e	0.1 ± 0.1b

Mean values within the same column followed by different letters are significantly different by Duncan's Multiple Range Test (DMRT) at p = 0.05. Assessment of germination and vigor index was done at day 9 after placing treated seeds for germination inside petri dishes with moist paper towels. Data presented here are mean values ± SE of at least three replications.

3.7 Effect of endophytic bacteria on shoot and root growth of *C. sativus*

The isolated endophytic bacteria had positive effects on growth and dry matter production of cucumber seedlings compared to untreated control (Table 2). The highest shoot length (5.2 ± 0.2 cm) was found in seedlings obtained from seeds treated with BBoS-1, which was statistically similar to the effects of *B. subtilis* BRtL-2 (4.7 ± 0.2 cm). The highest root length was recorded in BCL-1-treated (5.3 ± 0.5 cm) seedlings. The lowest shoot and root lengths were found in non-inoculated control (0.5 ± 0.0 and 2.7 ± 0.8 cm, respectively) seedlings, which were statistically lower than those from all other treatments.

The highest shoot fresh weight of cucumber seedlings was found in BBoS-1 (159.1 ± 1.9 mg) and the lowest was recorded in control (33.2 ± 0.6 mg), which was statistically different from the rest of the treatments (Table 2). The highest root fresh weight was found in cucumber seedlings obtained from seeds treated with *B. amyloliquifaciens* BDR-2 (36.5 ± 1.7 mg). The lowest root fresh weight was found in untreated control seedlings (16.5 ± 2.5 mg), which was statistically different from all other treated seedlings. The seedlings obtained from seeds treated with BRtL-3 had the highest shoot (19.8 ± 0.7 mg) and root dry weight (0.3 ± 0.1 mg), respectively, whereas untreated control had 8.4 ± 0.5 and 0.1 ± 0.1 mg/seedling dry weight, respectively.

3.8 Effect of endophytic bacteria on germination, shoot and root growth of rice

Similar to cucumber seedlings, endophytic bacteria remarkably enhanced shoot and root growth of rice seedlings (Figure 2). Rice seeds treated with three selected bacterial isolates, *B. subtilis* strain BRtL-2, BBoS-1 and BCL-1, had 100% germination. Root and shoot lengths were significantly enhanced by these three bacterial isolates compared to untreated control (Figure 2). The seedlings obtained from rice seeds treated with *B. subtilis* strain BRtL-2 had the highest shoot length (4.7 ± 0.6 cm) followed by BBoS-1 (4.0 ± 0.3 cm) and BCL-1 (3.9 ± 0.3 cm) (Figure 2A). The lowest shoot length was recorded in untreated control (2.9 ± 0.2 cm). A significantly longer

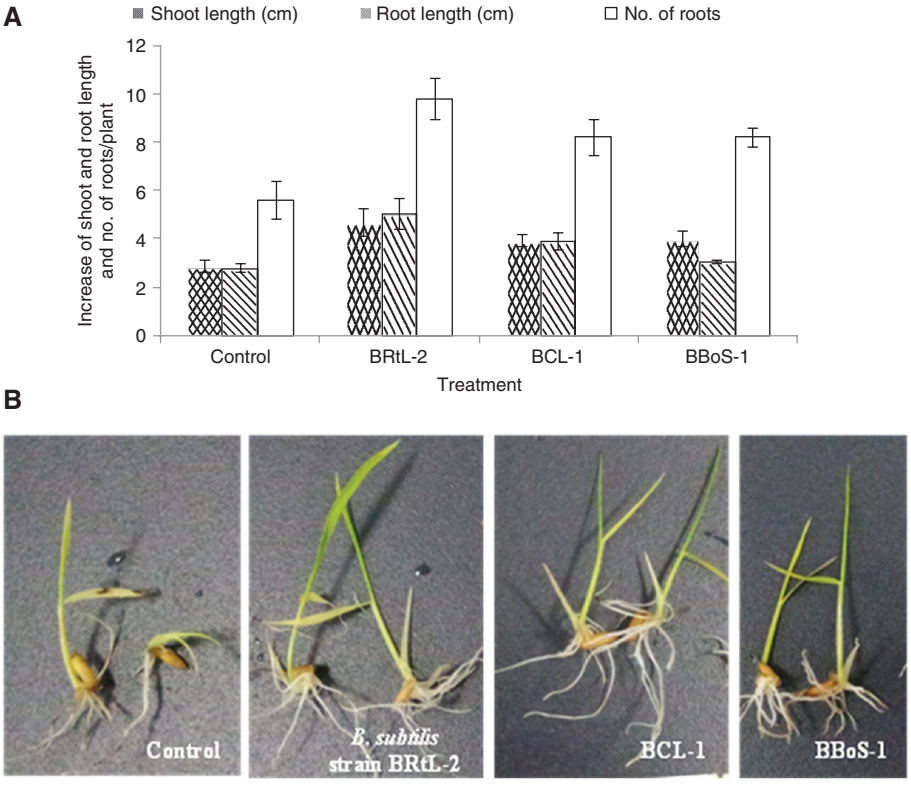

Figure 2: Effect of seed treatment with endophytic bacterial isolates on root and shoot growth of rice seedlings (A) and (B). (A) Graph showing endophytic bacterial treatments (X-axis) on the growth characteristics of rice seedlings (Y-axis) grown in laboratory conditions. Bars are the mean ± SE. (B) Pictures showing higher root and shoot growth in bacteria-treated seedlings compared to untreated control seedlings. Photographs were taken at day 9 after placement of treated seeds in petri dishes for germination.

(5.0 ± 0.6 cm) root system was found in seedlings of *B. subtilis* BRtL-2-treated seeds compared to untreated control (2.8 ± 0.2 cm), which was the lowest.

4 Discussion

The use of synthetic chemicals as fertilizers and pesticides not only increases the production cost of crop but also causes deleterious effects on the environment and health of humans and other organisms in the ecosystem. The application of novel strains of endophytic bacteria has been found as natural alternatives to synthetic agrochemicals and an essential component of sustainable agriculture [3, 9]. In the present study, we isolated and characterized seven plant growth-promoting endophytic bacteria from a few traditional medicinal plants of Bangladesh. Out of total 16 isolated and screened endophytic bacteria, three, namely BCL-1, BDR-2 and BRtL-2, significantly inhibited growth and sclerotia production of the phytopathogenic fungus *S. sclerotiorum* (Table 1; Figure 1). Seven isolates significantly ($p \leq 0.05$) enhanced germination of seeds, increased vigor index of seedling and promoted growth of cucumber and rice compared to untreated control (Table 2; Figure 2). Two potent plant growth-promoting endophytes were identified as *B. subtilis* BRtL-2 and *B. amyloliquefaciens* BDR-2. Promotion of plant growth and suppression of phytopathogens by endophytic *Bacilli* have previously been reported [29–31]. Both *B. subtilis* and *B. amyloliquefaciens* have been commercialized in many countries as biological (biocontrol and biostimulant) alternatives to synthetic pesticides [32]. To the best of our knowledge, this is the first report of isolation and characterization of plant growth-promoting and antagonistic endophytic bacteria under *Bacillus* genus from traditional herbal medicinal plants of Bangladesh.

One of the important findings of the current study is that the three isolates, namely BCL-1, BDR-2 and BRtL-2, significantly inhibited growth of hyphae (55%–69%) and production of sclerotia of an economically important fungal pathogen *S. sclerotiorum* (Table 1; Figure 1), and two out of these three isolates belonged to *Bacillus* spp., as revealed by 16S rRNA gene sequencing. Inhibition of hyphal growth of various fungi including *S. sclerotiorum* by several strains of *B. subtilis* and *B. amyloliquifaciens* has previously been reported [17, 29, 33–35]. Isolate BRD-2 also inhibited about 68% radial growth of rice sheath blight fungus *Rhizoctonia solani* (Sultana et al. unpublished personal communication). The production of diverse classes of secondary metabolites and lytic enzymes is considered as a chemical weapon of *Bacillus* species against phytopathogens [9, 13, 17, 34]. Another interesting finding of this study was characteristic morphological alterations in hyphae of *S. sclerotiorum* during interactions with *B. subtilis* BRtL-2, *B. amyloliquifaciens* BDR-2 and BCL-1, indicating production of metabolites involved with antibiosis [5, 17]. Suppression of wheat blast fungus *Magnaporthe oryzae Triticum* through antibiosis by plant endophytic bacteria has recently been reported [14]. Our findings suggest that BDR-2 and BRtL-2 isolated from the native medicinal plants could be used as biocontrol agents against major phytopathogens in rice and cucumber. Strains of endophytic bacteria from locally adapted plant species may have higher potential to survive and multiply when used as biostimulant or biopesticide through augmentative inoculation. Further study is needed to isolate and characterize the bioactive metabolites from these *Bacillus* species.

A total of seven endophytic bacteria (BRtL-2, BDR-2, BCL-1, BDL-1, BDR-1, BRtL-3 and BBoS-1) isolated in this study significantly increased seed germination, and enhanced vigor and growth of cucumber seedlings compared to untreated control (Table 2). Similarly, three isolates (BCL-1, BBoS-1 and *B. subtilis* strain BRtL-2) also enhanced shoot and root development in rice seedlings (Figure 2). All seven strains produced high amounts of IAA. These findings suggest that the production of IAA by these bacterial endophytes is linked with growth promotion of cucumber and rice seedlings although a quantitative relationship of IAA amount and growth promotion could not be established. Furthermore, we found that *B. amyloliquefaciens* strain BDR-2 remarkably promoted growth and increased grain yield of rice up to 21% in field trial under varying doses of fertilizer application to the soil (Sultana et al. unpublished personal communication). These results indicate that endophytic *Bacillus* spp. from medicinal plants can colonize and promote the growth of unrelated host plants. High plant colonization and promotion of growth of rice by the endophytic bacteria isolated from unrelated host willow have been reported [36]. Beneficial bacteria with potential to provide growth and yield enhancement of multiple crops and suppression of harmful microbes are major candidates for biological pesticides to be used for sustainable agriculture. The enhancement of plant growth by IAA-producing bacteria including members of *Bacillus* genus has previously been reported [3, 37, 38]. Synthesis of phytohormones triggers the activity of specific enzymes (e.g. α-amylase), which promote early germination and increase the availability of starch assimilation [39].

5 Conclusion

In this study, we isolated and partially characterized some plant growth-promoting endophytic bacteria from medicinal plants of Bangladesh. Two potential isolates were identified as *B. subtilis* BRtL-2 and *B. amyloliquifaciens* BDR-2 by 16S rRNA gene sequencing. These isolates significantly suppressed mycelial growth and sclerotia production of a phytopathogenic fungus *S. sclerotiorum*. In addition, cucumber and rice seed treatment with these endophytic bacteria significantly enhanced the percentage of seed germination and plant vigor likely due to the higher amount of IAA production. These findings indicate that two *Bacillus* spp. isolated from local medicinal plants can be used as biostimulant and biocontrol agents for sustainable production of cucumber and rice in an eco-friendly manner. Further studies are needed to determine the modes of action of these medicinal plant endophytes and also to test their efficacy as biostimulant and biocontrol agents in field conditions.

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References

1. Sturz AV, Christie BR, Nowak J. Bacterial endophytes: potential role in developing sustainable systems of crop production. Crit Rev Plant Sci 2000;19:1–302.
2. Khan MM, Haque E, Paul NC, Khaleque MA, Al-Garni SM, Rahman M, et al. Enhancement of growth and grain yield of rice in nutrient deficient soils by rice probiotic bacteria. Rice Sci 2017;24:264–73.
3. Rahman M, Sabir AA, Mukta JA, Khan MM, Mohi-Ud-Din M, Miah MG, et al. Plant probiotic bacteria *Bacillus* and *Paraburkholderia* improve growth, yield and content of antioxidants in strawberry fruit. Scientific Rep 2018;8:2504.
4. Hurek T, Reinhold-Hurek B. *Azoarcus* spp. strain BH72 as a model for nitrogen fixing grass endophytes. J Biotechnol 2003;106:169–78.
5. Islam MT, Hashidoko Y, Deora A, Ito T, Tahara S. Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne peronosporomycetes. Appl Environ Microbiol 2005;71:3786–96.
6. Islam MT, Hossain MM. Biological control of Peronosporomycete phytopathogens by bacterial antagonists. In: Maheshwari DK, editor. Bacteria in agrobiology: disease management. Berlin-Heidelberg: Springer-Verlag, 2013:167–218.
7. Zohara F, Akanda MA, Paul NC, Rahman M, Islam MT. Inhibitory effects of *Pseudomonas* spp. on plant pathogen *Phytophthora capsici* in vitro and in planta. Biocat Agric Biotechnol 2016;5:69–77.
8. Mukta JA, Rahman M, Sabir AA, Gupta DR, Surovy MZ, Rahman M, et al. Chitosan and plant probiotics application enhance growth and yield of strawberry. Biocat Agric Biotechnol 2017;11:9–18.
9. Islam MT, Rahman MM, Pandey P, Jha CK, Aeron A. Bacilli and agrobiotechnology. Switzerland: Springer International Publishing AG, 2017:1–416.
10. Kevin VJ. Plant growth promoting rhizobacteria as biofertilizer. Plant Soil 2003;255:571–86.
11. Yobo KS, Laing MD, Hunter CH. Effect of commercially available rhizobacteria strains on growth and production of lettuce, tomato and pepper. South Afr J Plant Soil 2004;21:230–5.
12. Khatun A, Farhana T, Sabir AA, Islam SM, West HM, Rahman M, et al. *Pseudomonas* and *Burkholderia* inhibit growth and asexual development of *Phytophthora capsici*. Z Naturforsch 2018;73:123–35.
13. Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, et al. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol 2007;25:1007–14.
14. Dutta S, Surovy MZ, Gupta DR, Mahmud NU, Chanclud E, Win J, et al. Genomic analyses reveal that biocontrol of wheat blast by *Bacillus* spp. may be linked with production of antimicrobial compounds and induced systemic resistance in host plants. 2018. https://doi.org/10.6084/m9.figshare.5852661.v1.
15. Lucangeli C, Bottini R. Effects of *Azospirillum* spp. on endogenous gibberellin content and growth of maize (*Zea mays* L.) treated with uniconazole. Symbiosis 1997;23:63–72.
16. Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 2009;321:305–39.
17. Islam MT, Hossain MM. Plant probiotics in phosphorus nutrition in crops, with special reference to rice. In: Maheshwari DK, editor. Bacteria in agrobiology: plant probiotics. Berlin, Heidelberg: Springer, 2012:325–63.
18. Purdy LH. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 1979;69:875–80.
19. Agrios GN. Plant pathology. San Diego, CA: Academic Press, 1997.
20. Bais HP, Fall R, Vivanco JM. Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol 2004;134:307–19.
21. Prova A, Akanda MA, Islam S, Sultana F, Islam MT, Hossain MM. First report of stem and pod blight of hyacinth bean caused by *Sclerotinia sclerotiorum*. J Plant Pathol 2014;96:607.
22. Somasegaran P, Hoben HJ. Handbook for rhizobia: methods in legume-rhizobium technology. New York: Springer-Verlag, 1994:332–41.
23. Bergey DH, Holt JG, Noel RK. Bergey's manual of systematic bacteriology, 9th ed. Baltimore, MD: Williams and Wilkins, 1994:1935–2045.

24. Hayward AC. A method for characterizing *Pseudomonas solanacearum*. Nature (London) 1960;186:405.
25. Rajat RM, Ninama GL, Mistry K, Parmar R, Patel K, Vegad MM. Antibiotic resistance pattern in *Pseudomonas aeruginosa* species isolated at a tertiary care hospital, Ahmadabad. National J Med Res 2012;2:156–9.
26. Deora A, Hashidoko Y, Islam MT, Tahara S. Antagonistic rhizoplane bacteria induce diverse morphological alterations in Peronosporomycete hyphae during in vitro interaction. Eur J Plant Pathol 2006;112:311–22.
27. Bric JM, Bostock RM, Silverstone SE. Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 1991;57:535–8.
28. Gordon SA, Weber RP. Colorimetric estimation of indole acetic acid. Plant Physiol 1951;26:192–5.
29. Fernando WG, Nakkeeran S, Zhang Y, Savchuk S. Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Prot 2007;26:100–7.
30. Rahman MM, Hossain DM, Suzuki K, Shiiya A, Suzuki K, Dey TK, et al. Suppressive effects of *Bacillus* spp. on mycelia, apothecia and sclerotia formation of *Sclerotinia sclerotiorum* and potential as biological control of white mold on mustard. Australasian Plant Pathol 2016;45:103–17.
31. Zhang JS, Xue AG. Biocontrol of sclerotinia stem rot (*Sclerotinia sclerotiorum*) of soybean using novel *Bacillus subtilis* strain SB24 under control conditions. Plant Pathol 2010;59:382–91.
32. Borriss R. Phytostimulation and biocontrol by plant-associated *Bacillus amyloliquefaciens* FZB42: an update. In: Islam MT, Rahman M, Pandey P, Jha CK, Aeron A, editors. Bacilli and agrobiotechnology. Switzerland: Springer International Publishing AG, 2017:163–84.
33. Expert JM, Digat B. Biocontrol of Sclerotinia wilt of sunflower by *Pseudomonas fluorescens* and *Pseudomonas putida* strains. Can J Microbiol 1995;41:685–91.
34. Mondol MA, Shin HJ, Islam MT. Diversity of secondary metabolites from marine *Bacillus* species: chemistry, biosynthesis and biological activity. Marine Drugs 2013;11:2846–72.
35. Ouhaibi-Ben Abdeljalil N, Vallance J, Gerbore J, Rey P, Daami-Remadi M. Bio-suppression of Sclerotinia stem rot of tomato and biostimulation of plant growth using tomato-associated rhizobacteria. J Plant Pathol Microbiol 2016;7:2.
36. Kandel SL, Hershberger N, Kim SH, Doty SL. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci 2015;55:1765–72.
37. Mishra M, Kumar U, Mishra PK, Prakash V. Efficiency of plant growth promoting rhizobacteria for the enhancement of *Cicer arietinum* L. growth and germination under salinity. Adv Biol Res 2010;4:92–6.
38. Rathaur P, Raja W, Ramteke PW, John SA. Effect of UV-B tolerant plant growth promoting rhizobacteria (PGPR) on seed germination and growth of *Withania somnifera*. Adv Appl Sci Res 2012;3:1399–404.
39. Gholami A, Shahsavani S, Nezarat S. The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. World Acad Sci Engin Technol 2009;3:25.