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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 of conducting research to discover, characterize and evaluate 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 also were 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 varying level compared to untreated controls. All isolates produced high levels of indole-3-acetic acid (6.3 to 63



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μg $mL^{-1}$) *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 using as natural plant growth promoter and biopesticides in sustainable crop production.

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

## 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 (PGPR) 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 improvement of crop yield, *B. subtilis* also induces resistance to the fungal phytopathogens. Strains of *B. amyloliquifaciens* 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 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 wide host range, which has been reported to infect 64 plant families [18, 19]. It causes white mold, cottony rot, watery





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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 field condition, yield loss may exceed 50% due to *Sclerotinia* blight in peanut under favorable environmental conditon. Control of plant diseases caused by *S. sclerotiorum* by synthetic fungicides is problematic due to high cost, low efficacy and deleterious effects to 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 in 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 evaluate 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 1706, 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 -1706, Bangladesh, which was previously isolated from hyacinth bean [21]. This organism was regularly cultured on Potato Dextrose Agar (PDA) medium and preserved in PDA slant.

### 2.2 Isolation of endophytic bacteria



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Sixteen endophytic bacteria were isolated from different well known medicinal plant parts such as root and leaf of *Duranta plumeri*, leaf of *Ocium gratissimum* L., leaf of *O. gratissimum* L., seed of *Terminalia bohera*, and leaf of *Manihot esculenta* from Dhaka, Gazipur and Rajshahi districts of Bangladesh. Five-gram fresh plant tissues from each of different organs was 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) 3 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 $1\times10^{-6}$ in SDW, from which100 µl was spread evenly on petri dishes containing yeast extract glucose agar (YGA) medium and incubated at 25 $^{o}$C for 24 h. Bacterial colonies were isolated based on the colony color, shape and other unique identification criterion, and purified by repeated streak plate culture. The purified single colony isolates were preserved in 20% glycerol solution at -20$^{o}$C for subsequent experiments.

**2.3 Morphological and biochemical characterization of endophytic bacterial isolates**

Morphological characteristics of colonies such as size, shape, color and growth pattern were recorded under light microscope after 24 h of growth on YGA medium at 25 ± 2 ºC 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.

**2.4 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 single bacterial colony were harvested from agar broth and re-suspended in 100 µl SDW by vortexing for 10s. For the determination of 16S rRNA sequences of active strains, chromosomal DNA extraction was done using commercial DNA extraction kit (ATP Biotech inc, Taiwan) and quantified comparing with lambda DNA marker after agarose gel electrophoresis. The 16S rRNA region was amplified by PCR using a universal primer 27F (5´-AGAGTTTGATCCTGGCTCAG-3´)



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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, 40s of denaturation at 94 °C, 40s 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 NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for DNA-DNA homology.

### 2.5 Determination of *S. sclerotiorum* growth suppression by endophytes

To assess antagonistic activities, 7 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 sterile cork borer and placed 3 cm apart from each endophyte colony on PDA plate [26]. 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].

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

where, X = Mycelial growth of the pathogen in absence of the antagonist; and Y= Mycelial growth of the pathogen in 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.

### 2.6 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, 1ml of $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$-fold diluted bacterial cells were added to the broth and incubated at 25 °C for 10 days. Before inoculation of bacterial



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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 3h, after which time mycelial dry weight was recorded individually to determine the effect of each antagonistic bacterial strain.

### 2.7 Plant growth promotion activity of the endophytic bacteria

#### 2.7.1 Indole-3-acetic acid production

For determination and quantification of indole-3-acetic acid (IAA) production, colonies of bacterial strains were inoculated into Jensen's broth (Sucrose 20 $gL^{-1}$, $K_2HPO_4$ 1 g $L^{-1}$, $MgSO_4$. $7H_2O$ 0.5 g $L^{-1}$, NaCl 0.5 g $L^{-1}$, $FeSO_4$ 0.1 g $L^{-1}$, $NaMoO_4$ 0.005 g $L^{-1}$, $CaCO_3$ 2 g $L^{-1}$) (Bric et al. 1991) containing 2 mg $mL^{-1}$L-tryptophan and incubated at 28 ± 2 °C with continuous shaking at 125 rpm for 48 h on a benchtop shaker (make and model). Two milliliter of culture solution was centrifuged at 12000 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 $FeCl_3.6H_2O$) and incubated for 20 min in darkness at room temperature as described by Gordon & 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 $ml^{-1}$). Supernatants from un-inoculated test tubes were used as control.

#### 2.7.2 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 15000 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 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 in room temperature to



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ensure better coating with bacteria. The number of bacterial cells per seed was ca. $1\times10^8$ CFU, counted by using serial dilution technique. Inoculated seeds were planted in pots containing soil mix. After germination, seedlings were watered on alternate days.

### 2.7.3 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 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 7th day.

Germination percentage and vigor index were calculated using following formula:

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

$$\text{Vigor Index} = \{\text{Mean shoot length (cm)} + \text{Mean root length (cm)}\} \times \text{Germination \%}$$

### 2.8 Statistical analysis

Data from different experiments were analyzed using statistical software SPSS (Version 15) and 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 yeast extract glucose agar 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



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gene bank. The 16S rRNA sequences of BRtL-2 and BDR-2 showed 100% similarity with *Bacillus subtilis* strain Tc1 (accession number GU391355.1) and *B. amyloliquifaciens* strain D41 (accession number KC441776.1), respectively. The isolates BRtL-2 and BDR-2 were isolated from two famous traditional medicinal plants namely, *Ocium gratissimum* L. (leaf) and *Duranta plumeri* (root).

### 3.2 Biochemical characterization of endophytic bacteria

Based on *in vitro* antagonism against *S. sclerotiorum* and ability to produce indole-3-acetic acid (IAA), 7 isolates namely, BDR-1 (root of *Duranta plumeri*), BDR-2 (root of *D. plumeri*), BDL-1 (leaf of *D. plumeri*), BRtL-2 (leaf of *Ocium gratissimum* L.), BRtL-3 (leaf of *O. gratissimum* L.), BBoS-1 (seed of *Terminalia bohera*) and BCL-1 (leaf of *Manihot 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 7 isolates reacted positively to the catalase test and Gram staining test except BRtL-3. Only 4 isolates (BDL-1, BDR-1, BDR-2 and BRtL-2) reacted positively to oxidase test. All but BRtL-3 reacted negatively to the KOH solubility test.

### 3.3 Indole-3-acetic acid (IAA) 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 to 63 µg $mL^{-1}$. The highest IAA was produced by isolate BRtL-3 (63 µg $mL^{-1}$) followed by BDL-1 (60.8 µg $mL^{-1}$), *B. amyloliquifaciens* BDR-2 (58.6 µg $mL^{-1}$) and BBoS-1 (52.3 µg $mL^{-1}$). However, the lowest IAA production (6.3 µg $mL^{-1}$) was found in *B. subtilis* BRtL-2. IAA produced by BDR-1 and BCL-1 were 8.6 and 12.6 µg $mL^{-1}$, 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*



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In a dual culture assay, hyphal growth of *S. sclerotiorum* towards 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. amyloliquifaciens* BDR-2 (66.7%) and *B. subtilis* BRtL-2 (54.7%). The percent of hyphal growth inhibition by the bacterial endophytes were statistically different from each other as well as untreated control plate (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 diameters, unusually long and pointed hyphal tips and hyphal lysis of *S. sclerotiorum* were observed microscopically in approaching hyphae towards 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 untreated control plate (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). Interaction of *S. sclerotiorum* with *B. amyloliquifaciens* BDR-2 resulted in hyper-branching, curly growth and break 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.

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

Mycelial growth of *S. sclerotiorum* was remarkably affected by 3 bacterial antagonists in 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, highest *S. sclerotiorum* mycelial dry weight (3.3 ± 0.01g) 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 *B. amyloliquifaciens* BDR-2 treated flask



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followed by 1.8 g and 2.4 g in strains *B. subtilis* BRtL-2 and BCL-1, treated flask, 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 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 with other treatments except BDR-1 (515.7 ± 25) but far higher than the untreated control (314.8 ± 30) (Table 2).

### 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 with effects of *B. subtilis* BRtL-2 (4.7 ± 0.2 cm). The highest root length was recorded in BCL-1 (5.3 ± 0.5 cm) treated seedlings. The lowest shoot and root length was found in non-inoculated control (0.5 ± 0.0 and 2.7 ± 0.8 cm, respectively) seedlings, which was statistically lower 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 mg and 0.1 ± 0.1 mg/seedling dry weight, respectively.

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



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Similar to cucumber seedlings, endophytic bacteria remarkably enhanced shoot and root growth of rice seedlings (Figure 2). Rice seeds treated with selected 3 bacterial isolates, *B. subtilis* strain BRtL-2, BBoS-1 and BCL-1 had 100% germination. Root and shoot length were significantly enhanced by these 3 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). Significantly longer (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

Use of synthetic chemicals as fertilizers and pesticides not only increase production cost of crop but also cause deleterious effects on the environment and health of humans and other organisms in the ecosystem. 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 viz. BCL-1, BDR-2 and BRtL-2 significantly inhibited growth and sclerotia production of the phytopathogenic fungus *S. sclerotiorum* (Table 1 and 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 three isolates viz. BCL-1, BDR-2 and BRtL-2 significantly inhibited growth of hyphae (55-69%) and production of sclerotia of an



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economically important fungal pathogen *S. sclerotiorum* (Table 1 and 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). Production of diverse classes of secondary metabolites and lytic enzymes are considered chemical weapons 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 have 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, 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 7 strains produced high amounts of indole-3-acetic acid (IAA). These findings suggest that 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 growth of unrelated host plants. High plant colonization and promotion of growth of rice by the endophytic bacteria isolated from unrelated



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host willow has 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. Enhancement of plant growth by IAA producing bacteria including members of *Bacillus* genus has previously been reported [3, 37, 38]. Synthesis of phytohormones trigger the activity of specific enzymes (e.g., α-amylase), that 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 % seed germination and plant vigor likely due to 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.

## Acknowledgement

We are thankful to the World Bank for financial support to CP # 2071of the Higher Education Quality Enhancement Project (CP # 2071) of University Grant Commission of Bangladesh. Sincere thanks are due to the Commonwealth Scholarship Commission, UK for partial funding to this work and Commonwealth Academic Staff Fellowship to MTI.

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Date: March 6, 2018

Editor-in-Chief
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**Manuscript ID**: ZNC.2018.0002
**Manuscript title**: Endophytic *Bacillus subtilis* and *B. amyloliquifaciens* from medicinal plants inhibit mycelial growth of *Sclerotinia sclerotiorum* and promote plant growth"

Dear Editor,

Thank you for the letter dated 27 February 2018. Based on the instructions provided in your letter, we uploaded the file of the revised manuscript on the Journal's Website. As you may notice, we have tried our best to revise the manuscript based on the valuable comments made by the knowledgeable reviewers. Accordingly, we have uploaded a copy of the revised version of the original manuscript. As significant time, will be required for the additional experiments for elucidation of detailed mechanisms, we wish to publish this short report with current data. As we only used 16S rRNA sequencing for the tentative identification of bacteria at species level and consid ering the brevity, we used *Bacillus* spp. is the revised title.

Appended to this letter is our point-by-point response to the comments raised by the editor and reviewers. We would like to take the opportunity to express our sincere thanks to the expert reviewers/editor who identified several areas in our manuscript that needed corrections as well as modifications.

Should you have any further queries regarding this revised version, please feel free to contact me.
I look forward to hearing from you soon.

Kind regards,

**M. Tofazzal Islam, Ph D**
(*Fellow of Fulbright, Commonwealth, JSPS and Alexander von Humboldt Foundation*)
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# Our Responses to the Comments of Reviewers

## Reviewer: 1

Comments to the Author
Few comments in the manuscript.

**Our reply:** Many thanks for considering our work for the publication in *Z. Naturforsch. C*. We thoroughly revised the manuscript for better expression and linguistic accuracy.

## Reviewer: 2

Comments to the Author
The authors have reported isolation of 7 endophytic bacteria from 4 different plants, and screened their ability to restrict growth of *Sclerotinia sclerotiorum* in vitro condition, and also determined the IAA production ability and enhancement of growth of rice seedlings and cucumber in pot trials.
**Comment 1:** There should be some experiments and description on mechanism of inhibitory effect of bacteria on target pathogen.

**Our reply:** Thank you for this valuable comment. Yes, we fully agree with this comment. The findings described in this report were to isolate and characterize potential plant growth promoting endophytic bacteria from medicinal plants and screen their effects on unrelated crop plant host. This paper describes findings of our first project. Elucidation of detail mechanisms of their action on plants were beyond the scope of that project. In a follow-up project, we tested two promising *Bacillus* spp. (BDR-2 and BRtL-2) on growth promotion and disease suppression at the field level and a detailed report including mechanisms will be published elsewhere followed by this primary article. In field trial, we found that the *Bacillus amyloliquefaciens* strain BDR-2 remarkably promote growth and increase grain yield of rice up to 21% depending on the nutrient status of soils. We added this information in the discussion section of current manuscript as data not shown. However, studies related to unravelling the mechanism are now underway and may take significant amount of time.

*Bacillus* spp. are well established as plant growth promoting bacteria and many of them are commercialized as biopesticides as they produce diverse arrays of antimicrobial compounds (please see more details in our recent book as http://www.springer.com/us/book/9783319444086).

2. How does endophytes of medicinal plants are supposed to increase growth of unrelated hosts (rice and cucumber)? this need to be addressed with suitable experiments. there is no experiment performed for colonization of endophyte on host plant.

**Our reply:** Thank for this valuable comment. Several lines of evidence suggest that some endophytic bacteria can highly colonize and remarkably promote growth and yield of unrelated host (please see Kandel et al. 2015, Crop Science 55, 1765-1772). Our two promising *Bacillus* sp. isolates produced IAA and suppressed a generalist fungal phytopathogen *Scleritinia sclerotiorum* in vitro. Involvement of growth promotion by the production of phytohormone IAA and suppression of disease through secretion of various antimicrobial substances by the *Bacillus* spp. have been reported in a large volume of literature. We mentioned earlier that we have field experimental data on these endophytes for promotion of growth and yield of rice, which will be reported elsewhere.

3. In pot trials, there is no treatment with pathogen, and so, claim of biocontrol is based on in vitro screening only. So, the findings remain inconclusive and can't be justified.

**Our reply:** In this study, we screened endophytic bacteria only in vitro bioassay. We are planning to conduct the greenhouse and field experiments for evaluating in vivo performances of selected antagonistic endophytes from this study.

4. Does the strain of *S. sclerotiorum* used in study was virulent against the variety used for cucumber and rice in pot trials? There is no trial taken up in pot trial with pathogen, which would had been useful to establish the role of bacteria in biocontrol.

**Our reply:** Yes, it's an important question. The strain of *Sclerotinia sclerotiorum* used in this study was isolated as an aggressive pathogen of hyacinth bean (please see Prova et al. 2014, Journal of Plant Pathology 96, 607). *S. sclerotiorum* is the most nonspecific, dangerous phytopathogen which has been reported to infect 64 plant families including Cucurbitaceae (Purdy 1979, Phytopathology 69, 875-880). It also causes Sclerotinia stem rot disease in cucumber. We recently found that BDR-2 and BRtL-2 also remarkably suppress *Rhizoctonia solani*, which is an important phytopathogen of rice.

5. Photomicrographs are of poor quality and does not offer any evidence. SEM images may provide better support to the scientific claims.

**Our reply:** We are sorry for the low resolution of the images in the pdf version. We added higher resolution images. The SEM images will obviously give more insight, however, unfortunately, we have no facility for taking SEM images.

6. IAA producing Bacillus spp are well known as plant growth stimulant, and therefore, authors may be advised to present the novelty of this work.

**Our reply:** Yes, growth promotion of plants by the strains of Bacillus spp. are well-established. Among various mechanisms IAA production is a prominent one. Our work for the first time isolated and characterized two promising plant growth promoting isolates of the genus *Bacillus* from the native medicinal plants, which have high potential for practical use as biostimulants and biocontrol agents. We have already got field trial data on this hypothesis in a separate project which will be published elsewhere after the publication of these preliminary findings.

7. Some conclusive experiments are required to understand the mechanism of growth inhibition of target pathogen, which is missing from the manuscript.

**Our reply:** Yes, elucidation of mechanisms of plant growth promotion and suppression of growth of phytopathogen was beyond the scope of this report. However, in discussion section we added information of our field trial with BDR-2 which stimulated growth and increased yield by 21% compared to untreated control.

8. Table 1,2,4 are not required and the information may be given in text itself. Fig 2a, what was 'number of roots', and how does number of roots correlate to growth enhancement? need to be clarified.
I still see there is lot of room for improvement in the manuscript, and it need more experiments to be performed before reaching any conclusion.

**Our reply:** Yes, we fully agree with this suggestion for the improvement of the manuscript. Tables 1, 2, and 4 were deleted and information are given in the text. Root morphology plays a crucial role in nutrient uptake by the plants. Higher number of lateral roots induced by the endophyte might be linked with the potential for higher nutrient uptake (please see our recent articles on stimulation of root growth by Bacillus was correlated with higher growth and yield of strawberries at https://www.nature.com/articles/s41598-018-20235-1

We sincerely appreciate the reviewers for valuable critical comments for improvement of this manuscript. We have tried our level best to revise the manuscript towards expected improvement. As we only used 16S rRNA sequencing for the tentative identification of bacteria at species level and considering the brevity, we used *Bacillus* spp. is the revised title.

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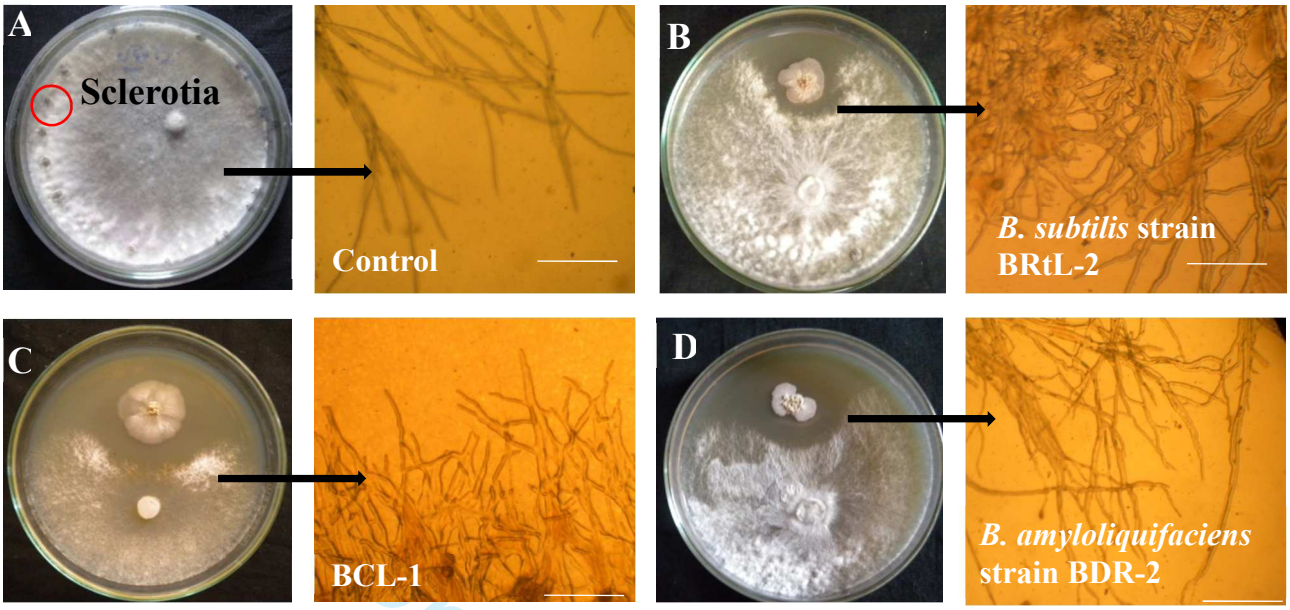


Figure 1. Interactions between antagonistic *Bacillus* spp. and *S. sclerotiorum* in dual culture assay in PDA and photo microscopic view of morphological alterations in *S. sclerotiorum* hyphae A to D. A. Normal mycelia with sclerotia (round red mark) of *S. sclerotiorum* in untreated control; B. excessive 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 *B. amyloliquifaciens* strain BDR-2; Scale bars = 100 µm

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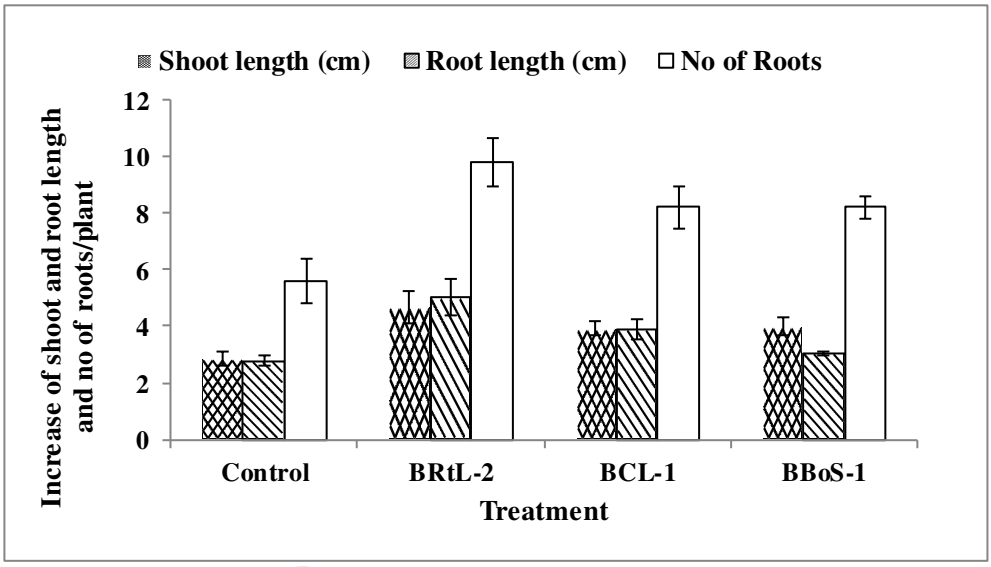
(A)




(B)


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 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. photos were taken at day 9 after placement of treated seeds in Petri dishes for germination

**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 | % mycelial growth inhibition of *S. sclerotiorum* | Mycelial dry weight after 10 days | % 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 |
| *B. 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 $p = 0.05$. [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**.** Data presented here are mean value ± SE of at least three replications.

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**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.

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