



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

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

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22 **Abstract:** Plant growth promoting bacteria that are also capable of suppressing plant pathogenic fungi
23 play an important role in sustainable agriculture. There is a critical need of conducting research to
24 discover, characterize and evaluate efficacy of new strains of such bacteria in controlling highly
25 aggressive plant pathogens. In this study, we isolated endophytic bacteria from medicinal plants of
26 Bangladesh and evaluated their antagonistic capacity against an important phytopathogenic fungus
27 *Sclerotinia sclerotiorum*. Growth promoting effects of those isolates on cucumber and rice seedlings also
28 were assessed. Among 16 morphologically distinct isolates, BDR-2, BRtL-2, and BCL-1 significantly
29 inhibited the growth of *S. sclerotiorum* through induction of characteristic morphological alterations in
30 hyphae and reduction of mycelial dry weight. When cucumber and rice seeds were treated with these
31 endophytic bacteria, seven isolates (BCL-1, BDL-1, BRtL-2, BRtL-3, BDR-1, BDR-2 and BBoS-1)
32 enhanced seed germination, seedling vigor, seedling growth, and number of roots per plant at varying
33 level compared to untreated controls. All isolates produced high levels of indole-3-acetic acid (6.3 to 63

34 $\mu\text{g mL}^{-1}$) *in vitro*. Two most potential isolates, BDR-2 and BRtL-2 were identified as *Bacillus*
35 *amyloliquefaciens* and *B. subtilis*, respectively based on the 16S rRNA gene sequencing. These results
36 suggest that endophytic *Bacillus* species from native medicinal plants have great potential for using as
37 natural plant growth promoter and biopesticides in sustainable crop production.

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

41 1 Introduction

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

49 Among various reported plant growth promoting and biocontrol bacteria, species of *Bacillus*
50 showed the highest potential both *in vitro* and *in vivo* [9]. For example, *B. subtilis* produces
51 various phytohormones such as indole-3-acetic acid (IAA), cytokinins, zeatin, gibberellic acid
52 and abscisic acid that are transported into the shoot through the xylem, delay senescence and thus
53 boost production of lettuce, tomato, cucumber and pepper [10, 11]. Apart from improvement of
54 crop yield, *B. subtilis* also induces resistance to the fungal phytopathogens. Strains of *B.*
55 *amyloliquefaciens* and *B. subtilis* are known to competitively colonize plants and can
56 simultaneously act as biofertilizer and antagonists (biopesticides) of bacteria, fungi,
57 peronosporomycetes and nematodes [12-14]. Colonization of cucumber plants by *B. polymyxa*
58 increased enzymatic activities with concurrent increase in cucumber yield up to 25% compared
59 to non-treated control [15]. Production of growth stimulating phytohormones, solubilization and
60 mobilization of phosphates, production of siderophores, production of antibiotics, inhibition of
61 plant ethylene synthesis and induction of plant systemic resistance to pathogens are considered
62 as the mechanisms of plant growth promotion by endophytic bacteria [2, 3, 13, 16, 17].

63 *Sclerotinia sclerotiorum* is the most destructive phytopathogen with wide host range, which
64 has been reported to infect 64 plant families [18, 19]. It causes white mold, cottony rot, watery

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3 65 soft rot, stem rot, drop, crown rot and twig blight and many other serious diseases in plants
4 66 resulting in significant yield losses worldwide [18]. In field condition, yield loss may exceed
5 67 50% due to *Sclerotinia* blight in peanut under favorable environmental conditon. Control of plant
6 68 diseases caused by *S. sclerotiorum* by synthetic fungicides is problematic due to high cost, low
7 69 efficacy and deleterious effects to the environment [19]. Biological agents can be the best
8 70 alternatives for controlling plant diseases due to the reasons mentioned above [5, 7, 20].
9 71 Bangladesh is rich in diversity of medicinal plants from which discovery of novel endophytic
10 72 bacteria to use in biopesticides against notorious phytopathogens including *S. sclerotiorum*
11 73 holds great potential. No systematic study has so far been conducted on isolation and
12 74 identification of endophytic bacteria from medicinal plants of Bangladesh, and evaluate their
13 75 effects on growth promotion and protection of plants from phytopathogens. Therefore, the
14 76 objectives of this study were to (i) isolate and identify potential endophytic bacteria from some
15 77 important medicinal plants; (ii) evaluate inhibitory effects of the isolated endophytic bacteria
16 78 against *S. sclerotiorum*, and (iii) assess the effects of endophytes on seed germination, seedling
17 79 vigor and growth of rice and cucumber.
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31 81 **2 Materials and methods**

32 82 33 83 **2.1 General experimental procedure**

34 84 All the chemicals and reagents used in this study were available at the department of
35 85 Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706,
36 86 Bangladesh, previously procured from Merck AG, Germany. All photographs were taken by a
37 87 high-resolution camera attached to a compound microscope.
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44 88 45 89 **2.2 Source and preservation of *Sclerotinia sclerotiorum* strain**

46 90 The pathogen *S. sclerotiorum* was collected from the stock culture of the Department of Plant
47 91 Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur -1706,
48 92 Bangladesh, which was previously isolated from hyacinth bean [21]. This organism was
49 93 regularly cultured on Potato Dextrose Agar (PDA) medium and preserved in PDA slant.
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54 94 55 95 **2.2 Isolation of endophytic bacteria**

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3 96 Sixteen endophytic bacteria were isolated from different well known medicinal plant parts such
4 as root and leaf of *Duranta plumeri*, leaf of *Ocimum gratissimum* L., leaf of *O. gratissimum* L.,
5 97 seed of *Terminalia bohera*, and leaf of *Manihot esculenta* from Dhaka, Gazipur and Rajshahi
6 98 districts of Bangladesh. Five-gram fresh plant tissues from each of different organs was washed
7 99 under running tap water and surface sterilized with 70% ethanol for 10 min followed by 1%
8 100 NaOCl for 1 min and finally washed with sterile distilled water (SDW) 3 times to remove excess
9 101 NaOCl [2]. Tissue pieces were then smashed in a sterilized mortar and pestle. Smashed plant
10 102 materials were serially diluted up to 1×10^{-6} in SDW, from which 100 μ l was spread evenly on
11 103 petri dishes containing yeast extract glucose agar (YGA) medium and incubated at 25 °C for 24
12 104 h. Bacterial colonies were isolated based on the colony color, shape and other unique
13 105 identification criterion, and purified by repeated streak plate culture. The purified single colony
14 106 isolates were preserved in 20% glycerol solution at -20°C for subsequent experiments.
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109 2.3 Morphological and biochemical characterization of endophytic bacterial isolates

110 Morphological characteristics of colonies such as size, shape, color and growth pattern were
111 recorded under light microscope after 24 h of growth on YGA medium at 25 ± 2 °C as described
112 earlier [22]. A series of biochemical tests were performed to characterize the isolated endophytic
113 bacteria using the protocols described in Bergey's Manual of Systematic Bacteriology [23]. For
114 the KOH solubility test, endophytic bacteria were mixed aseptically with 3% KOH solution on a
115 clean slide with an inoculating wire loop for 1 min and observed for the formation of a thread-
116 like mass. Catalase and oxidase tests were performed as described earlier by Hayward [24] and
117 Rajat et al. [25], respectively.
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119 2.4 Molecular identification of isolated endophytic bacteria

120 Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed followed
121 by sequencing to identify the active bacterial strains [7]. Initially, cells from single bacterial
122 colony were harvested from agar broth and re-suspended in 100 μ l SDW by vortexing for 10s.
123 For the determination of 16S rRNA sequences of active strains, chromosomal DNA extraction
124 was done using commercial DNA extraction kit (ATP Biotech inc, Taiwan) and quantified
125 comparing with lambda DNA marker after agarose gel electrophoresis. The 16S rRNA region
126 was amplified by PCR using a universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3')

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3 127 and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR amplification was carried out in
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5 128 Mastercycler® Gradient (Eppendorf, Hamburg, Germany) for 30 successive cycles consisting of
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7 129 5 min of initial denaturation at 94 °C, 40s of denaturation at 94 °C, 40s of annealing at 55 °C, and
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9 130 1 min of extension at 72 °C with a final extension for 10 min at 72 °C. PCR mix was purified and
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11 131 analyzed followed by sequencing with Big Dye terminator cycle sequencing ready reaction kits
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13 132 (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequences were combined
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14 133 using the Laser gene version 7.1 program. The 16S rRNA gene sequences of the strains were
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16 134 then subjected to BLAST search using NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for
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18 135 DNA-DNA homology.
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20 137 **2.5 Determination of *S. sclerotiorum* growth suppression by endophytes**

21 138 To assess antagonistic activities, 7 bacterial strains were tested against *S. sclerotiorum*
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23 139 using a dual culture assay. A 6-mm diameter mycelial plug of the pathogen was cut from an
24
25 140 actively growing colony with sterile cork borer and placed 3 cm apart from each endophyte
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27 141 colony on PDA plate [26]. Inhibition zone was observed after incubation at 25 °C for 72 h. The
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29 142 radial growth of the pathogen and percentage inhibition was calculated after 10 days as described
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31 143 by Zohara et al. [7].

$$32 \quad 144 \quad \% \text{ Inhibition of growth} = \frac{X - Y}{X} \times 100$$

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35 145 where, X = Mycelial growth of the pathogen in absence of the antagonist; and Y= Mycelial
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37 146 growth of the pathogen in presence of the antagonist
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40 148 Morphological characters of approaching hyphae were observed under a light microscope and
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42 149 images were recorded with a digital camera attached with the microscope. At least five samples
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44 150 were examined for each combination of the pathogen and antagonistic endophyte.
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46 151 47 152 **2.6 Effect of endophytic bacterial strains on mycelial dry weight of *S. sclerotiorum***

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49 153 Quantitative antagonistic effect of isolated endophytic bacterial strains was measured. Briefly, a
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51 154 block of *S. sclerotiorum* was grown in a conical flask containing 200 ml potato dextrose broth
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53 155 (PDB) for 3 days. After 3 days, 1ml of 1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 -fold diluted bacterial
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55 156 cells were added to the broth and incubated at 25 °C for 10 days. Before inoculation of bacterial

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3 157 strains, mycelial dry weight of control flasks was measured to determine whether bacterial
4 158 strains can decompose and decrease mycelial growth of *S. sclerotiorum*. After 10 days of
5 159 pathogen mycelia and bacteria interaction, mycelia from each flask were placed on separate filter
6 160 paper and allowed to dry for 3h, after which time mycelial dry weight was recorded individually
7 161 to determine the effect of each antagonistic bacterial strain.
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13 163 **2.7 Plant growth promotion activity of the endophytic bacteria**

14 164 15 165 **2.7.1 Indole-3-acetic acid production**

16 166 For determination and quantification of indole-3-acetic acid (IAA) production, colonies of
17 167 bacterial strains were inoculated into Jensen's broth (Sucrose 20 g L⁻¹, K₂HPO₄ 1 g L⁻¹, MgSO₄.
18 168 7H₂O 0.5 g L⁻¹, NaCl 0.5 g L⁻¹, FeSO₄ 0.1 g L⁻¹, NaMoO₄ 0.005 g L⁻¹, CaCO₃ 2 g L⁻¹) (Bric et
19 169 al. 1991) containing 2 mg mL⁻¹ L-tryptophan and incubated at 28 ± 2 °C with continuous shaking
20 170 at 125 rpm for 48 h on a benchtop shaker (make and model). Two milliliter of culture solution
21 171 was centrifuged at 12000 g for 1 min, and 1 ml aliquot of the supernatant was mixed with 2 ml of
22 172 Salkowski's reagent (150 ml conc. H₂SO₄, 250 ml distilled water, 7.5 ml 0.5 M FeCl₃.6H₂O) and
23 173 incubated for 20 min in darkness at room temperature as described by Gordon & Weber [28].
24 174 IAA production was indicated by the development of a pink-red color, and the absorbance was
25 175 measured at 530 nm using a spectrophotometer (PD-303U, APEL, Japan). The concentration of
26 176 IAA was determined using a standard curve prepared from pure IAA solutions (0, 5, 10, 15, 20,
27 177 25, 30, 35, 40, 45, 50, 55, 60 and 65 µg ml⁻¹). Supernatants from un-inoculated test tubes were
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42 180 **2.7.2 Preparation of bacterial inocula for seed treatment**

43 181 Bacterial strains that were isolated from medicinal plants were cultured separately in conical
44 182 flasks containing 200 ml yeast peptone broth and incubated in an orbital shaker at 120 rpm for 72
45 183 h at 25 °C for preparation of inocula. Bacterial cells were collected via centrifugation at 15000
46 184 rpm for 1 min at 4 °C. After washing with SDW, bacterial pellets were suspended in 0.6 ml
47 185 SDW, vortexed and used for seed treatment. One gram cucumber and rice seeds were surface
48 186 sterilized with 70% ethanol for 10 min followed by washing with SDW. Seeds were then soaked
49 187 in bacterial suspension in a petri dish (for how long?) and dried overnight in room temperature to
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188 ensure better coating with bacteria. The number of bacterial cells per seed was ca. 1×10^8 CFU,
189 counted by using serial dilution technique. Inoculated seeds were planted in pots containing soil
190 mix. After germination, seedlings were watered on alternate days.

191 192 **2.7.3 Effect of bacterial seed treatment on germination and vigor index of cucumber and** 193 **rice**

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

199 Germination percentage and vigor index were calculated using following formula:

$$200 \quad \text{Germination (\%)} = (\text{No of seeds germinated} / \text{Total no of seeds sown}) \times 100$$

$$201 \quad \text{Vigor Index} = \{ \text{Mean shoot length (cm)} + \text{Mean root length (cm)} \} \times \text{Germination \%}$$

202 203 204 **2.8 Statistical analysis**

205 Data from different experiments were analyzed using statistical software SPSS (Version 15) and
206 Microsoft Office Excel 2007 program and XL STAT (Version. 2012). Fisher's protected LSD
207 test was used to determine the levels of significant differences among the mean values at $P \leq$
208 0.05. The experimental design was completely randomized, consisting of at least three
209 replications for each treatment and were repeated at least twice.

210 211 **3 Results**

212 **3.1 Isolation, screening and identification of endophytic bacteria**

213 A total of 16 endophytic bacteria were isolated and purified from medicinal plants by repeated
214 streak plate culture on yeast extract glucose agar medium. Initially, all strains were screened
215 against *S. sclerotiorum* in a dual culture agar plate for assessing their antagonistic activities.
216 Three bacterial strains namely BCL-1, BRtL-2 and BDR-2 out of 16 showed vigorous but
217 varying level of hyphal growth inhibition against *S. sclerotiorum*. Ribosomal gene sequences
218 (16S rRNA) of two most potent antagonists were done for identification and deposited in the

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, *Ocimum 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 *Ocimum 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 $\mu\text{g mL}^{-1}$. The highest IAA was produced by isolate BRtL-3 (63 $\mu\text{g mL}^{-1}$) followed by BDL-1 (60.8 $\mu\text{g mL}^{-1}$), *B. amyloliquifaciens* BDR-2 (58.6 $\mu\text{g mL}^{-1}$) and BBoS-1 (52.3 $\mu\text{g mL}^{-1}$). However, the lowest IAA production (6.3 $\mu\text{g mL}^{-1}$) was found in *B. subtilis* BRtL-2. IAA produced by BDR-1 and BCL-1 were 8.6 and 12.6 $\mu\text{g mL}^{-1}$, 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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3 250 In a dual culture assay, hyphal growth of *S. sclerotiorum* towards the colonies of *B.*
4 251 *amyloliquifaciens* BDR-2, *B. subtilis* BRtL-2 and BCL-1 was remarkably inhibited compared
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6 252 with the untreated control plate (Figure 1A-D). Clear inhibition zones formed on the agar
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8 253 medium due to the interaction between antagonistic bacteria and *S. sclerotiorum*. The largest
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10 254 inhibition of radial growth was recorded in BCL-1 (69.1%) followed by *B. amyloliquifaciens*
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12 255 BDR-2 (66.7%) and *B. subtilis* BRtL-2 (54.7%). The percent of hyphal growth inhibition by the
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14 256 bacterial endophytes were statistically different from each other as well as untreated control plate
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16 257 (Table 1). No sclerotia were produced in plates treated with bacterial endophytes, whereas
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18 258 sclerotia were plentiful in the control plates.

19 259 Distinct morphological alterations such as irregular and excessive branching, abnormal swelling
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21 260 of hyphal diameters, unusually long and pointed hyphal tips and hyphal lysis of *S. sclerotiorum*
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23 261 were observed microscopically in approaching hyphae towards the colonies of bacterial
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25 262 endophytes (BCL-1, BRtL-2 and BDR-2) (Figure 1B-D). Normal polar growth of hyphae was
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27 263 observed under a microscope in untreated control plate (Figure 1A). Interactions with *B. subtilis*
28
29 264 BRtL-2 caused disruption of normal radial growth of hyphae by inducing excessive branching,
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31 265 curling, swelling and pointed hyphae (Figure 1B). Stunted mycelial growth with swelling,
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33 266 pointed tips and excessive branching was observed in *S. sclerotiorum* due to interaction with
34
35 267 endophytic bacterial strain BCL-1 (Figure 1C). Interaction of *S. sclerotiorum* with *B.*
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37 268 *amyloliquifaciens* BDR-2 resulted in hyper-branching, curly growth and break down of hyphae
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39 269 (Figure 1D). Although excessive branching and loss of polar growth in *S. sclerotiorum* were
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41 270 common, each strain in addition induced characteristic morphological alterations, indicating
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43 271 production of diverse inhibitory compounds by the bacterial antagonists.

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273 **3.5 Reduction of mycelial dry weight of *S. sclerotiorum* by the endophytic bacteria**

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

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3 280 followed by 1.8 g and 2.4 g in strains *B. subtilis* BRtL-2 and BCL-1, treated flask, respectively
4 281 (Table 1).
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8 283 **3.6 Effect of endophytic bacteria on seed germination and vigor index of *Cucumis sativus***

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10 284 All treatments improved seed germination and vigor index of cucumber seedlings compared to
11
12 285 untreated control. Hundred percent germination of cucumber seeds was obtained in all bacterial
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14 286 isolates used in seed treatment except BBoS-1 treated seeds ($98.3 \pm 2.8\%$). Lowest germination
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16 287 percentage ($96.7 \pm 2.8\%$) was recorded in untreated control (Table 2). Seedlings obtained from
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18 288 endophyte treated seeds showed enhanced plant vigor index compared to untreated control.
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20 289 Treatment with BRtL-3 showed the highest vigor index of (871.6 ± 40), which was statistically
21
22 290 similar with other treatments except BDR-1 (515.7 ± 25) but far higher than the untreated control
23
24 291 (314.8 ± 30) (Table 2).
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27 293 **3.7 Effect of endophytic bacteria on shoot and root growth of *C. sativus***

28
29 294 The isolated endophytic bacteria had positive effects on growth and dry matter production of
30
31 295 cucumber seedlings compared to untreated control (Table 2). The highest shoot length (5.2 ± 0.2
32
33 296 cm) was found in seedlings obtained from seeds treated with BBoS-1, which was statistically
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35 297 similar with effects of *B. subtilis* BRtL-2 (4.7 ± 0.2 cm). The highest root length was recorded in
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37 298 BCL-1 (5.3 ± 0.5 cm) treated seedlings. The lowest shoot and root length was found in non-
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39 299 inoculated control (0.5 ± 0.0 and 2.7 ± 0.8 cm, respectively) seedlings, which was statistically
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41 300 lower from all other treatments.

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43 301 The highest shoot fresh weight of cucumber seedlings was found in BBoS-1 (159.1 ± 1.9 mg)
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45 302 and the lowest was recorded in control (33.2 ± 0.6 mg), which was statistically different from the
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47 303 rest of the treatments (Table 2). The highest root fresh weight was found in cucumber seedlings
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49 304 obtained from seeds treated with *B. amyloliquifaciens* BDR-2 (36.5 ± 1.7 mg). The lowest root
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51 305 fresh weight was found in untreated control seedlings (16.5 ± 2.5 mg), which was statistically
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53 306 different from all other treated seedlings. The seedlings obtained from seeds treated with BRtL-3
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55 307 had the highest shoot (19.8 ± 0.7 mg) and root dry weight (0.3 ± 0.1 mg), respectively whereas
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57 308 untreated control had 8.4 ± 0.5 mg and 0.1 ± 0.1 mg/seedling dry weight, respectively.
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60 310 **3.8 Effect of endophytic bacteria on germination, shoot and root growth of rice**

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3 311 Similar to cucumber seedlings, endophytic bacteria remarkably enhanced shoot and root growth
4 312 of rice seedlings (Figure 2). Rice seeds treated with selected 3 bacterial isolates, *B. subtilis* strain
5 313 BRtL-2, BBoS-1 and BCL-1 had 100% germination. Root and shoot length were significantly
6 314 enhanced by these 3 bacterial isolates compared to untreated control (Figure 2). The seedlings
7 315 obtained from rice seeds treated with *B. subtilis* strain BRtL-2 had the highest shoot length ($4.7 \pm$
8 316 0.6 cm) followed by BBoS-1 (4.0 ± 0.3 cm) and BCL-1 (3.9 ± 0.3 cm) (Figure 2A). The lowest
9 317 shoot length was recorded in untreated control (2.9 ± 0.2 cm). Significantly longer (5.0 ± 0.6 cm)
10 318 root system was found in seedlings of *B. subtilis* BRtL-2 treated seeds compared to untreated
11 319 control (2.8 ± 0.2 cm), which was the lowest.
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21 321 **4 Discussion**

22 322 Use of synthetic chemicals as fertilizers and pesticides not only increase production cost of crop
23 323 but also cause deleterious effects on the environment and health of humans and other organisms
24 324 in the ecosystem. Application of novel strains of endophytic bacteria has been found as natural
25 325 alternatives to synthetic agrochemicals and an essential component of sustainable agriculture [3,
26 326 9]. In the present study, we isolated and characterized seven plant growth promoting endophytic
27 327 bacteria from a few traditional medicinal plants of Bangladesh. Out of total 16 isolated and
28 328 screened endophytic bacteria, three viz. BCL-1, BDR-2 and BRtL-2 significantly inhibited
29 329 growth and sclerotia production of the phytopathogenic fungus *S. sclerotiorum* (Table 1 and
30 330 Figure 1). Seven isolates significantly ($P \leq 0.05$) enhanced germination of seeds, increased vigor
31 331 index of seedling and promoted growth of cucumber and rice compared to untreated control
32 332 (Table 2; Figure 2). Two potent plant growth promoting endophytes were identified as *B. subtilis*
33 333 BRtL-2 and *B. amyloliquefaciens* BDR-2. Promotion of plant growth and suppression of
34 334 phytopathogens by endophytic *Bacilli* have previously been reported [29-31]. **Both *B. subtilis***
35 335 **and *B. amyloliquefaciens* have been commercialized in many countries as biological (biocontrol**
36 336 **and biostimulant) alternatives to synthetic pesticides [32]. To the best of our knowledge this is**
37 337 **the first report of isolation and characterization of plant growth promoting and antagonistic**
38 338 **endophytic bacteria under *Bacillus* genus from traditional herbal medicinal plants of Bangladesh.**

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40
41 339 One of the important findings of the current study is that three isolates viz. BCL-1, BDR-2
42 340 and BRtL-2 significantly inhibited growth of hyphae (55-69%) and production of sclerotia of an
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3 341 economically important fungal pathogen *S. sclerotiorum* (Table 1 and Figure 1) and two out of
4 342 these three isolates belonged to *Bacillus* spp. as revealed by 16S rRNA gene sequencing.
5 343 Inhibition of hyphal growth of various fungi including *S. sclerotiorum* by several strains of *B.*
6 344 *subtilis* and *B. amyloliquifaciens* has previously been reported [17, 29, 33-35]. Isolate BRD-2
7 345 also inhibited about 68% radial growth of rice sheath blight fungus *Rhizoctonia solani* (Sultana
8 346 et al. unpublished personal communication). Production of diverse classes of secondary
9 347 metabolites and lytic enzymes are considered chemical weapons of *Bacillus* species against
10 348 phytopathogens [9, 13, 17, 34]. Another interesting finding of this study was characteristic
11 349 morphological alterations in hyphae of *S. sclerotiorum* during interactions with *B. subtilis* BRtL-
12 350 2, *B. amyloliquifaciens* BDR-2 and BCL-1 indicating production of metabolites involved with
13 351 antibiosis [5, 17]. Suppression of wheat blast fungus *Magnaporthe oryzae* *Triticum* through
14 352 antibiosis by plant endophytic bacteria have recently been reported [14]. Our findings suggest
15 353 that BDR-2 and BRtL-2 isolated from the native medicinal plants could be used as biocontrol
16 354 agents against major phytopathogens in rice and cucumber. Strains of endophytic bacteria from
17 355 locally adapted plant species may have higher potential to survive and multiply when used as
18 356 biostimulant or biopesticide through augmentative inoculation. Further study is needed to isolate
19 357 and characterize the bioactive metabolites from these *Bacillus* species.

20 358 A total of seven endophytic bacteria (BRtL-2, BDR-2, BCL-1, BDL-1, BDR-1, BRtL-3, and
21 359 BBoS-1) isolated in this study significantly increased seed germination, enhanced vigor and
22 360 growth of cucumber seedlings compared to untreated control (Table 2). Similarly, three isolates
23 361 (BCL-1, BBoS-1 and *B. subtilis* strain BRtL-2) also enhanced shoot and root development in rice
24 362 seedlings (Figure 2). All 7 strains produced high amounts of indole-3-acetic acid (IAA). These
25 363 findings suggest that production of IAA by these bacterial endophytes is linked with growth
26 364 promotion of cucumber and rice seedlings although a quantitative relationship of IAA amount
27 365 and growth promotion could not be established. Furthermore, we found that *B.*
28 366 *amyloliquifaciens* strain BDR-2 remarkably promoted growth and increased grain yield of rice
29 367 up to 21% in field trial under varying doses of fertilizer application to the soil (Sultana et al.
30 368 unpublished personal communication). These results indicate that endophytic *Bacillus* spp. from
31 369 medicinal plants can colonize and promote growth of unrelated host plants. High plant
32 370 colonization and promotion of growth of rice by the endophytic bacteria isolated from unrelated

371 **host willow has been reported** [36]. Beneficial bacteria with potential to provide growth and
372 yield enhancement of multiple crops and suppression of harmful microbes are major candidates
373 for biological pesticides to be used for sustainable agriculture. Enhancement of plant growth by
374 IAA producing bacteria including members of *Bacillus* genus has previously been reported [3,
375 37, 38]. Synthesis of phytohormones trigger the activity of specific enzymes (e.g., α -amylase),
376 that promote early germination and increase the availability of starch assimilation [39].

377

378 **5 Conclusion**

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

390

391 **Acknowledgement**

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4 Date: March 6, 2018
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6 Editor-in-Chief
7 Zeitschrift für Naturforschung C
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10 **Manuscript ID:** ZNC.2018.0002

11 **Manuscript title:** Endophytic *Bacillus subtilis* and *B. amyloliquifaciens* from medicinal plants
12 inhibit mycelial growth of *Sclerotinia sclerotiorum* and promote plant growth"
13
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15 Dear Editor,
16

17 Thank you for the letter dated 27 February 2018. Based on the instructions provided in
18 your letter, we uploaded the file of the revised manuscript on the Journal's Website. As you may
19 notice, we have tried our best to revise the manuscript based on the valuable comments made by
20 the knowledgeable reviewers. Accordingly, we have uploaded a copy of the revised version of
21 the original manuscript. As significant time, will be required for the additional experiments for
22 elucidation of detailed mechanisms, we wish to publish this short report with current data. As we
23 only used 16S rRNA sequencing for the tentative identification of bacteria at species level and consid
24 ering the brevity, we used *Bacillus* spp. is the revised title.
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32 Appended to this letter is our point-by-point response to the comments raised by the
33 editor and reviewers. We would like to take the opportunity to express our sincere thanks to the
34 expert reviewers/editor who identified several areas in our manuscript that needed corrections as
35 well as modifications.
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41 Should you have any further queries regarding this revised version, please feel free to contact
42 me.
43

44 I look forward to hearing from you soon.
45
46

47 Kind regards,
48
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50

51 **M. Tofazzal Islam, Ph D**

52 (*Fellow of Fulbright, Commonwealth, JSPS and Alexander von Humboldt Foundation*)

53 Professor

54 Department of Biotechnology
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For Review Only

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>).

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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 *Sclerotinia 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.

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4 **Our reply:** We are sorry for the low resolution of the images in the pdf version. We added higher
5 resolution images. The SEM images will obviously give more insight, however, unfortunately, we have
6 no facility for taking SEM images.
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10 6. IAA producing *Bacillus* spp are well known as plant growth stimulant, and therefore, authors may
11 be advised to present the novelty of this work.
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14 **Our reply:** Yes, growth promotion of plants by the strains of *Bacillus* spp. are well-established.
15 Among various mechanisms IAA production is a prominent one. Our work for the first time isolated
16 and characterized two promising plant growth promoting isolates of the genus *Bacillus* from the
17 native medicinal plants, which have high potential for practical use as biostimulants and biocontrol
18 agents. We have already got field trial data on this hypothesis in a separate project which will be
19 published elsewhere after the publication of these preliminary findings.
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26 7. Some conclusive experiments are required to understand the mechanism of growth inhibition of
27 target pathogen, which is missing from the manuscript.
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30 **Our reply:** Yes, elucidation of mechanisms of plant growth promotion and suppression of growth of
31 phytopathogen was beyond the scope of this report. However, in discussion section we added
32 information of our field trial with BDR-2 which stimulated growth and increased yield by 21%
33 compared to untreated control.
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38 8. Table 1,2,4 are not required and the information may be given in text itself. Fig 2a, what was
39 'number of roots', and how does number of roots correlate to growth enhancement? need to be
40 clarified.
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42 I still see there is lot of room for improvement in the manuscript, and it need more experiments to
43 be performed before reaching any conclusion.
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46 **Our reply:** Yes, we fully agree with this suggestion for the improvement of the manuscript. Tables 1,
47 2, and 4 were deleted and information are given in the text. Root morphology plays a crucial role in
48 nutrient uptake by the plants. Higher number of lateral roots induced by the endophyte might be
49 linked with the potential for higher nutrient uptake (please see our recent articles on stimulation of
50 root growth by *Bacillus* was correlated with higher growth and yield of strawberries at
51 <https://www.nature.com/articles/s41598-018-20235-1>
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4 We sincerely appreciate the reviewers for valuable critical comments for improvement of this
5 manuscript. We have tried our level best to revise the manuscript towards expected improvement. As
6 we only used 16S rRNA sequencing for the tentative identification of bacteria at species level and
7 considering the brevity, we used *Bacillus* spp. is the revised title.
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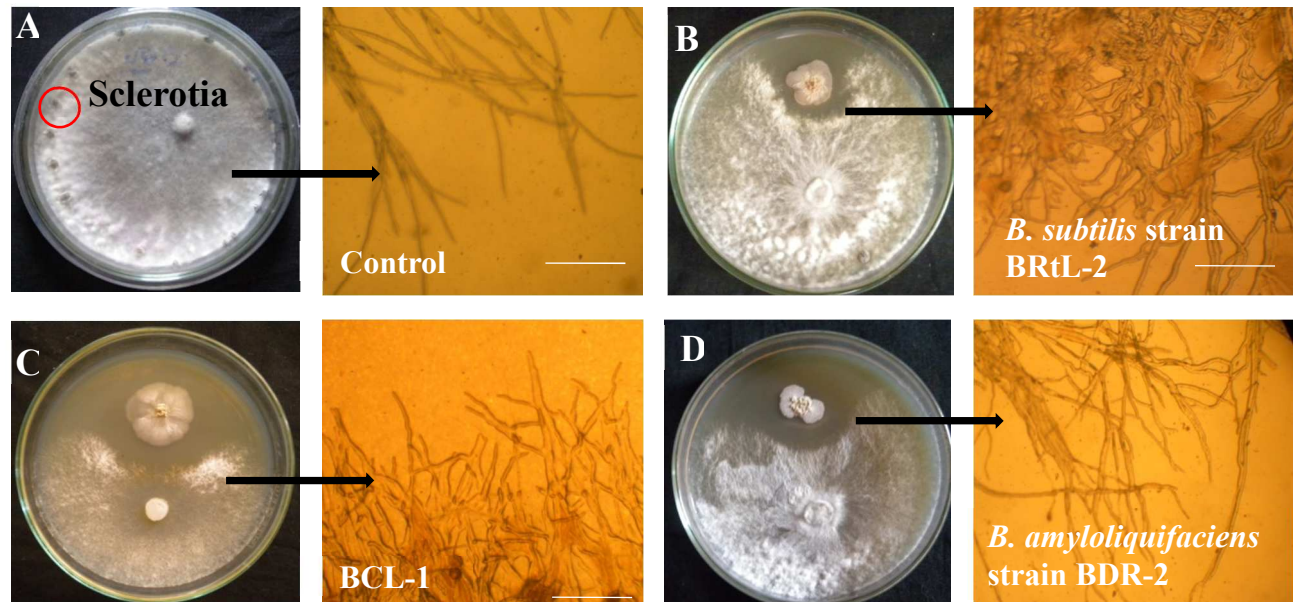
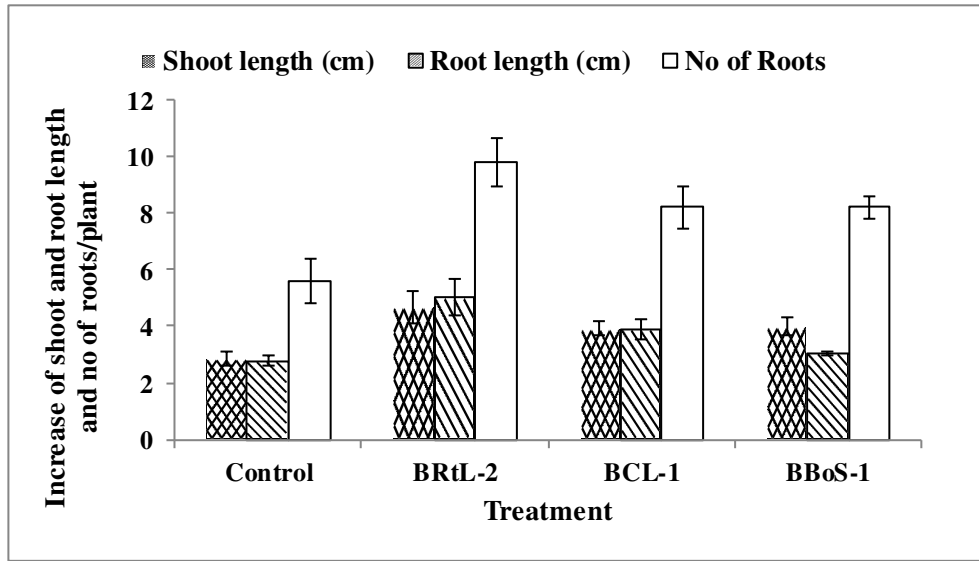


Figure 1. Interactions between antagonistic *Bacillus* spp. and *S. sclerotiorum* in dual culture assay in PDA and photo microscopic view of morphological alterations in *S. sclerotiorum* hyphae A to D. A. Normal mycelia with sclerotia (round red mark) of *S. sclerotiorum* in untreated control; B. excessive 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 μ m

(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 \pm 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 <i>S. sclerotiorum</i> (cm)	Sclerotial Number	% mycelial growth inhibition of <i>S. sclerotiorum</i>	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
<i>Bacillus subtilis</i> strain BRtL-2	2.0 ± 0.03b	0 ± 0	54.7 ± 0.6b	1.8 ± 0.01	46.3
<i>B. amyloliquifaciens</i> 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* ^bmycelial 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.

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.