

Molecular Identification and Evaluation of Indigenous Bacterial Isolates for Their Plant Growth Promoting and Biological Control Activities against Fusarium Wilt Pathogen of Tomato

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In search of an effective biological control agent against the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*, rhizospheric soil samples were collected from eight agro-ecological zones of Bangladesh. Among the bacteria isolated from soil, 24 isolates were randomly selected and evaluated for their antagonistic activity against *F. oxysporum* f. sp. *lycopersici*. The two promising antagonistic isolates were identified as *Brevundimonas olei* and *Bacillus methylotrophicus* based on morphological, biochemical and molecular characteristics. These two isolates were evaluated for their biocontrol activity and growth promotion of two tomato cultivars (cv. Pusa Rubi and Ratan) for two consecutive years. Treatment of Pusa Rubi and Ratan seeds with *B. olei* prior to inoculation of pathogen caused 44.99% and 41.91% disease inhibition respectively compared to the untreated but pathogen-inoculated control plants. However, treatment of Pusa Rubi and Ratan seeds with *B. methylotrophicus* caused 24.99% and 39.20% disease inhibition respectively. Furthermore, both the isolates enhanced the growth of tomato plants. The study re-

vealed that these indigenous bacterial isolates can be used as an effective biocontrol agent against Fusarium wilt of tomato.

Keywords : biological control, *Fusarium oxysporum*, fusarium wilt, plant growth promoting rhizobacteria, tomato

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Among the fungal diseases of tomato, Fusarium wilt is one of the most serious ones throughout the world, especially in Bangladesh. This disease is caused by soil-borne pathogenic fungi *F. oxysporum* f. sp. *lycopersici*. Fusarium wilt of tomato causes 10-90% yield loss in temperate region (Singh and Kamal, 2012). It has been estimated that this pathogen is responsible for 60-70% yield loss of tomato in Bangladesh (Raihan et al., 2016). The control of Fusarium wilt of tomato is difficult due to the ability of this pathogen to proliferate within vascular tissues of the host. Fungicides cannot effectively control this soil-borne pathogen. Moreover, application of fungicides may also kill some other microorganisms beneficial to tomato plants. In order to address such problems, in an environment friendly manner, biological control agents can be employed.

Earlier studies demonstrated that some microorganisms inhabiting in the rhizosphere region have the potentiality to enhance plant growth (Ge et al., 2016; Guo et al., 2004). Bacterial isolates from local agro-ecological environment and rhizosphere of specific plant is likely to be well adapted and perform better in this respect (Whipps, 1997). In order to get desired result, it is important to identify and use region-specific new microbial strains which can be used as potential biocontrol agents and plant growth promoters. Information pertaining to the use of rhizosphere inhabiting bacterial isolates from different agro-ecological regions of

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Bangladesh and their application for biological control is inadequate, particularly in case of tomato. Therefore, the present study was conducted to find novel indigenous rhizospheric bacterial isolates from different agro-ecological zones (AEZs) of Bangladesh and to evaluate their biocontrol potential against Fusarium wilt of tomato and their plant growth promoting ability.

Materials and Methods

Collection of soil sample. In order to collect indigenous biocontrol agents, plant rhizosphere soil samples were collected from fields of four different crops growing in eight different agro-ecological zones (AEZs) of Bangladesh (Table 1). The soil samples were collected from rhizosphere of rice (*Oryza sativa* L.), jute (*Corchorus capsularis* L.), bean (*Lablab niger* Medikus) and sesban (*Sesbania bispinosa* (Jacq.) W. Wight, following standard methods (Malleswari and Bagyanarayana, 2013).

Isolation and selection of bacteria. One gram of each of the collected soil samples was weighed and placed in the test tube containing 9 ml of 0.9% saline (Bahig et al., 2008). Ten folds serial dilution was made and an aliquot was spread onto nutrient agar (NA) plates. The plates were incubated at $28 \pm 2^\circ\text{C}$ for 24 h. The individual colonies formed on the NA plates were picked up with sterilized loop and transferred to fresh NA plates for further purification and isolation of single colony. The plates were incubated at $28 \pm 2^\circ\text{C}$ for 24 h. The single colonies developed separately at the last tip of the streaks were transferred to NA slants to serve as pure culture.

Morphological and growth characteristics of the isolates. Colonies of all the isolated rhizobacterial isolates formed on NA plates were evaluated for size, pigmentation, form, margin, elevation as described in Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 2000). Furthermore, tolerances of bacteria to pH, salinity and varying temperatures were evaluated using a turbidometric method (Chookietwattana and Maneewan, 2012).

Phytopathogen. The soil-borne pathogen *F. oxysporum* used in this study were obtained from the Plant Pathology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh.

Screening of the isolates for biocontrol potential. All the 24 isolates were assayed *in vitro* for interactions with soil-borne phytopathogen *F. oxysporum* on PDA media. Bioassay was studied by dual culture technique as per Ramathan et al. (2002). Five millimeters mycelial discs were cut from young growing edge of the fungus from seven days old culture with a sterilized cork borer and placed at one side of a Petri plate. The 24 bacterial isolates were streaked aseptically parallel to the fungus at a distance of 15-20 mm and incubated at $28 \pm 2^\circ\text{C}$ for 10 days. Three replications were maintained for each isolate. The inhibition zone between the two cultures was measured in nearest millimeter. After 10 days incubations the percent inhibition of the fungus was calculated by using the formula:

$$\text{Growth inhibition (\%)} = \frac{R1-R2}{R1} \times 100$$

Table 1. Location of the agricultural fields for the collection of rhizospheric soil samples

Rhizosphere soil source	Code name (No. of isolates)	Place of collection (Thana, District)	Agro-Ecological Zones (AEZ) covered
Rice (<i>Oryza sativa</i> L.)	Prk1, Prk2, Prk3, Prk4 (4)	Kumarkhali, Kushtia	High Ganges river floodplain
	Prd2, Prd5 (2)	Shotogram, Dinajpur	Old Himalayan piedmont plain
	Prju2, Prju3, Prju3b (3)	Jahangirnagar University Campus, Savar, Dhaka	Madhupur tract
	Prsp1, Prsp2, Prsp3 (3)	Jhinaigathi, Sherpur	Northern and eastern piedmont plains
Bean (<i>Lablab niger</i> Medik)	Prbo, Prbo4, Prbo5 (3)	Ujirpur, Barisal	Low high Ganges river floodplain
	Prb1, Prb2 (2)	Bogra sadar, Bogra	Level barind tract
Jute (<i>Corchorus capsularis</i> L.)	Pbbb1 (1)	Nobinagar, Brahmanbaria	Old Meghna estuarine floodplain
	Pbm3 (1)	Gouripur, Mymensingh	Old Brahmaputra floodplain
Sesban (<i>Sesbania bispinosa</i> (Jacq.) W. Wight)	Pjbb, Pjbb1, Pjbb3, Pjbb5 (4)	Nobinagar, Brahmanbaria	Old Meghna estuarine floodplain
	Pdbb (1)	Nobinagar, Brahmanbaria	Old Meghna estuarine floodplain

Where

R_1 = Radius (mm) of the fungus from the center of the colony towards the centre of the plate in the absence of antagonistic bacteria

R_2 = Radius (mm) of the fungus from center of the colony towards the antagonistic bacteria

Biochemical tests. On the basis of consistent antagonism and stress tolerance activities, two bacterial isolates Prb1 and Prd2 showing promising activity compared to the control were selected for further characterization based on standard biochemical tests (Schaad et al., 2001). The tests conducted were Gram reaction, catalase activity, oxidase activity, nitrate reduction, arginine decarboxylase activity, gelatin liquefaction, urease activity, levan formation from sucrose and utilization of glucose, maltose, lactose, xylose and mannitol. Results of these tests were scored as either positive or negative.

Production of extracellular hydrolytic enzymes such as amylase, protease and cellulase were tested by growing each bacterial isolate on the medium containing enzyme substrate; skim milk for protease assay (Naik et al., 2008), carboxymethyl cellulose for cellulase assay (Bhakthavathalu et al., 2013) and starch for amylase assay (Shruti et al., 2013). Solid agar plates amended with 0.44% of L-glycine was used for qualitative hydrogen cyanide production following the method of Bakker and Schippers (1987). The colour change of the filter paper from yellow to light brown, moderate brown or strong brown was examined for putative HCN production. Siderophore production was detected according to Naik et al. (2008). For assessment of growth on nitrogen free media Norris agar plates was used as described by Kumar et al. (2012).

Molecular identification. The molecular identification of two isolates was carried out by the 16S rRNA gene sequencing and subsequent analysis using NCBI-BLAST database (Altschul et al., 1990). For genomic DNA isolation, the selected two isolates were grown to log phase. The cells were harvested by centrifugation and genomic DNA was isolated using Maxwell 16 automated DNA extractor (USA) and quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). The 16S rRNA gene of each of the isolates were amplified by PCR using the primers 27F(AGAGTTTGATCMTGGCTCAG) and 1492R (GGTACCTTGTTACGACTT) according to Sudini et al. (2011). The PCR products were analyzed on 1% agarose gel and desired band was excised and purified. The PCR product was sequenced in both directions at 1st Base Laboratories, Malaysia using an automated DNA sequencer

(Genetic Analyzer ABI 3500, Thermo Fisher Scientific Inc., USA). The resulting DNA sequence was compared by NCBI-BLAST database (Altschul et al., 1990). Based on the highest degree of similarity, identity for the bacterial isolates was assigned.

For phylogenetic analysis, the 16S rRNA gene sequences were aligned with the Clustal W program and the tree was constructed with the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) integrated in the MEGA7 software (Kumar et al., 2016). The phylogenetic tree was tested with 1,000 bootstrap replicates.

Survival of the isolates. The two bacterial isolates were grown in nutrient broth medium and stored at constant temperature ($28 \pm 2^\circ\text{C}$). The samples taken from the growth medium and diluted to appropriately. The diluted cell suspensions (100 μl) were inoculated onto the solid NA media. The plates were incubated at ($28 \pm 2^\circ\text{C}$) temperature for 24 h. The number of colony forming units (cfu) was counted after one day of incubation at each week up to 35 days.

Effect of ferric ion on antagonistic activity. In order to determine the threshold level of iron at which siderophore biosynthesis is repressed, the biological agents were inoculated in King's B medium supplemented with different concentrations of FeCl_3 (0, 50, 70 and 100 μM). After inoculation, antagonism was observed following the method of Akköprü and Demir (2005).

Root colonization bioassay. Root colonization bioassay was carried out following the procedure described by Silva et al. (2003). Surface sterilized tomato seeds were soaked in 25 ml of bacterial suspension ($\text{OD}_{600} = 0.1$) and then transferred to sterile 0.6% water agar tubes. The seedlings were allowed to grow at room temperature ($28 \pm 2^\circ\text{C}$). Visual observations were performed daily in order to detect bacterial growth around arising roots.

Interaction studies of bacteria with other non-target soil beneficial organisms. The two selected bacterial isolates namely, Prb1 and Prd2 were tested for their interaction with other beneficial microorganism *B. subtilis*, *Trichoderma harzianum* and *T. reesei*. Pure culture of *B. subtilis*, *T. harzianum*, *T. reesei* obtained from the Plant Pathology Laboratory, Department of Botany, JU. Each of these three cultures were inoculated at a distance of 2 cm with respective two bacterial isolates and observed for the inhibition zone. The inoculated plates were observed for growth at regular intervals.

Effect of selected bacterial isolates on disease control and plant growth parameters of tomato.

Experiment was conducted to observe the effect of selected bacterial isolates on disease control and growth promotion of tomato plant in pot culture condition. Two most popular cultivars of tomato (cv. Ratan and Pusa Ruby) in Bangladesh were used in this study (Khalequzzaman et al., 2002; Mistry et al., 2008). For seed surface sterilization and water formulated bacterial inoculation, the method described by Weller and Cook (1983) was followed. For seed treatment, cell suspension containing approximately 1×10^8 cfu/ml was applied to surface sterilized seeds. Seedling vigor test in the laboratory was performed using the standard roll towel method (ISTA, 1999). After 10 days of incubation, observation pertaining to (a) germination (b) dead seed (c) seedling weight (d) root length (e) shoot length and (f) vigor index were recorded.

Germination percentage was calculated by using the formula (ISTA, 1976):

$$\text{Percentage of germination} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds taken for germination}} \times 100$$

Vigor of the seedling was determined by using the following formula (ISTA, 1996):-

$$\text{Vigor Index} = (\text{Mean of root length} + \text{Mean of shoot length}) \times \% \text{ of Seed germination}$$

Pot culture experiment was conducted during tomato growing season in 2015-16 and 2016-17 to assess interaction between the bacterial isolates and the targeted wilt pathogen. Soil used in the pots were homogenized and sterilized following the method described by Khalequzzaman et al. (2002). Treated seeds were permitted to dry for 6 hrs before planting. There were three replications of each treatment and the pots were arranged in randomized manner. Inoculations of plants with pathogen were performed after 3 weeks of sowing. Seedlings were inoculated with the spore and cell suspension of *F. oxysporum* by the soil-soak method (Tans-Kersten et al., 2001). The experimental setup included the following treatments: (1) non-infested soil (control), (2) soil treated with *F. oxysporum* only, (3) *F. oxysporum* + Prd2, (4) *F. oxysporum* + Prb1, (5) Prd2 only and (6) Prb1 only. The disease incidence was recorded up to 30 days after pathogen inoculation. To find out disease incidence of Fusarium wilt of tomato, method described by Song et al. (2004) was followed. Percent Disease Index (PDI) was calculated by the following formula:

$$\text{PDI} = \frac{S (\text{Disease scale} \times \text{plant number in that class})}{\text{Highest scale} \times \text{total number of plants}} \times 100$$

Biological control efficacy (BCE) was calculated according to Guo et al. (2004):

$$\text{BCE} = (D_c - D_t / D_c) \times 100\%$$

Where, D_c disease of control,

D_t disease of the treatment)

The observations with respect to the growth parameters including plant height, number of leaves, leaf area (Car-massi et al., 2007), shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, chlorophyll and carotenoid content were recorded. The estimation of chlorophyll a, b content was determined according to Maclachlan and Zalik (1963):

$$\text{Chlorophyll a} = \frac{(12.3 \times \text{OD663a} - 0.86 \times \text{OD645a})}{d \times 1000 \times W} \times V$$

$$\text{Chlorophyll b} = \frac{(19.3 \times \text{OD645a} - 0.86 \times \text{OD663a})}{d \times 1000 \times W} \times V$$

$$\text{Total Chlorophyll} = \frac{(20.2 \times \text{OD645}) + (8.02 \times \text{OD663})}{d \times 1000 \times W} \times V$$

The estimation of total carotenoids was done according to Ayvaz et al. (2012):

$$\text{Total carotenoid (mg)/g tissue} = 4.07 \times (\text{OD}_{450}) - (0.0435 \times \text{Chl}_a) + (0.367 \times \text{Chl}_b)$$

Statistical analysis. Data were analyzed for significant mean differences using two-way analysis of variance (ANOVA). Means were separated using Duncan's multiple range test (DMRT; $P = 0.05$) using SPSS software.

Results

Isolation and screening of bacterial isolates. In this work, a total of 24 bacteria were isolated from different plant rhizosphere soils from eight agro-ecological zones (AEZ) of Bangladesh (Table 1). The isolates varied in size, shape, elevation and pigment production capacity. These isolates were evaluated for their antagonistic and stress tolerance capacity.

In vitro antagonistic potential of isolated bacteria against *Fusarium oxysporum*. In PDA medium growth inhibition was found to range from 0.00 to 87.66% after

Table 2. *In vitro* biocontrol activity of bacterial isolates against *Fusarium oxysporum*

Test isolates	Radial growth of the pathogen (mm)	Inhibition over control (%)	Test isolates	Radial growth of the pathogen (mm)	Inhibition over control (%)
Prju2	5.66 ± 0.33 ^{ij}	79.03	Prk1	8.66 ± 0.33 ^{gh}	67.92
Pjbb1	7.33 ± 0.66 ^{hi}	72.85	Prk2	12.33 ± 0.88 ^{ef}	54.33
Pbm3	8.33 ± 0.88 ^{gh}	69.14	Prbo5	9.33 ± 0.57 ^{sh}	65.44
Pdbb	5 ± 1.52 ^{ij}	81.48	Prbo4	10.66 ± 0.88 ^{fg}	60.51
Prb1	3.33 ± 0.33 ^j	87.66	Prbo	19 ± 0.57 ^d	29.62
Pbbb1	5 ± 0.57 ^{ij}	81.48	Prsp1	23.33 ± 2.40 ^c	13.59
Pjbb5	13.66 ± 0.33 ^e	49.4	Prd5	12.66 ± 0.33 ^{ef}	53.11
Prju3b	7.33 ± 0.33 ^{hi}	72.85	Prju3	25 ± 0.33 ^c	7.40
Pjbb	12.66 ± 0.33 ^{ef}	53.11	Prsp2	20 ± 0.33 ^d	25.92
Prb2	12 ± 0.57 ^{ef}	55.55	Prsp3	28.33 ± 0.88 ^b	-
Prd2	7.33 ± 0.33 ^{hi}	72.85	Prk3	33.33 ± 0.88 ^a	-
Pjbb3	7.33 ± 0.88 ^{hi}	72.85	Control	27 ± 0.66 ^b	-
PrK4	9 ± 0.57 ^{gh}	66.66			

Values are the means of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level.

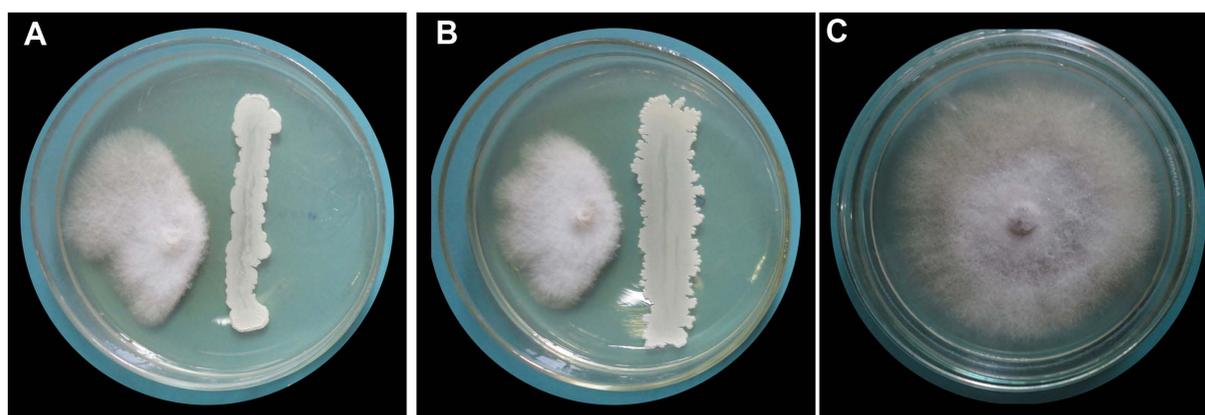


Fig. 1. Inhibition of *F. oxysporum* f. sp. *lycopersici* by two bacterial isolates (A) *Bacillus methylotrophicus* Prb1, and (B) *Brevundimonas olei* Prd2, and (C) mycelial growth of *F. oxysporum* in control plate.

10 days of incubation (Table 2). The bacterial isolate Prb1 exhibited maximum growth inhibition (87.66%) against *F. oxysporum*. Two potential bacterial isolates Prb1 (87.66%) and Prd2 (72.85%) showing consistent antagonistic (Fig. 1) and promising stress tolerance activities (data not shown) were selected for further characterization.

Biochemical characteristics of the bacterial isolates.

Several biochemical tests have been conducted to identify the biocontrol agents. The results of the biochemical tests have been summarized in Table 3.

Molecular identification. The PCR amplification of the 16S rRNA gene resulted in products approximately 1.5 kb in size. The obtained 16S rRNA gene sequences were deposited in GenBank under the accession number

MH458892 and MH458893 for the isolate Prd2 and Prb1, respectively. The BLAST searches revealed that the rRNA gene of the bacterial isolate Prd2 has 100% identity to that of *Brevundimonas olei* strains and Prb1 showed 100% identity to that of *Bacillus methylotrophicus* strains. The phylogenetic relation of the isolates is presented in Fig. 2. The results of molecular analyses were consistent with the morphological, biochemical and physiological traits of the isolates.

Survival profile for selected two bacterial isolates. The study showed the decrease in cfu of the isolate *B. olei* Prd2 after 28 days, while decrease in cfu was observed after 21 days in *B. methylotrophicus* Prb1 isolate (Table 4). It can be concluded that biocontrol agent Prd2 selected in this study can be preserved efficiently at least for 28 days at 28°C.

Table 3. Biochemical and physiological characteristics of two biocontrol agents

No.	Test name	Bacterial strain	
		Prb1	Prd2
1.	Gram reaction	+	-
2.	Oxidase test	+	+
3.	Catalase test	+	+
4.	Gelatin liquefaction	+	+
5.	Nitrate reduction	-	+
6.	Arginine decarboxylase activity	+	+
7.	Levan formation	+	-
8.	Urease test	+	+
9.	Carbon sources utilization:		
	Glucose	+	-
	Maltose	-	+
	Lactose	-	-
	Xylose	+	+
	Mannitol	-	-
10.	Starch hydrolysis test	+	+
11.	Protease activity	-	+
12.	Cellulase activity	-	+
13.	HCN production	-	+
14.	Siderophore production	+	+
15.	Growth on nitrogen free medium	-	+
16.	Root colonization test	+	+

All the tests were conducted in three replicates, '+' indicates positive reaction, '-' indicates negative reaction.

Table 4. Survival of selected bacterial isolates at an interval of 7 days

Days	Prd2 (cfu/ml)	Prb1 (cfu/ml)
0	1.05×10^6	1.56×10^6
7	6×10^6	14×10^6
14	5.2×10^7	8.8×10^7
21	1.38×10^8	9×10^7
28	1.65×10^8	3×10^6
35	7.4×10^7	-

Role of siderophore and iron deprivation in the *in vitro* inhibition of *F. oxysporum*. In the *in vitro* study aiming at whether this antagonistic effect was caused by the siderophore mechanism or not, the selected two bacterial isolates grown in iron-deprived and iron added medium at different ferric ion concentrations (Table 5). Siderophore mediated inhibition of fungal growth ranged from of 56.95 to 59.75% in the absence of FeCl_3 whereas the inhibition percentage ranged from 2.57 to 71.62% in presence of FeCl_3 . At 100 μM FeCl_3 , the two isolates induced the maximum inhibition.

Compatibility test. Results showed that none of the bacterial isolates (*B. methylotrophicus* Prb1 and *B. oley* Prd2) inhibited *B. subtilis*, *T. harzianum* and *T. reesei* in any of the plates; all the two bacterial isolates were compatible (data not shown).

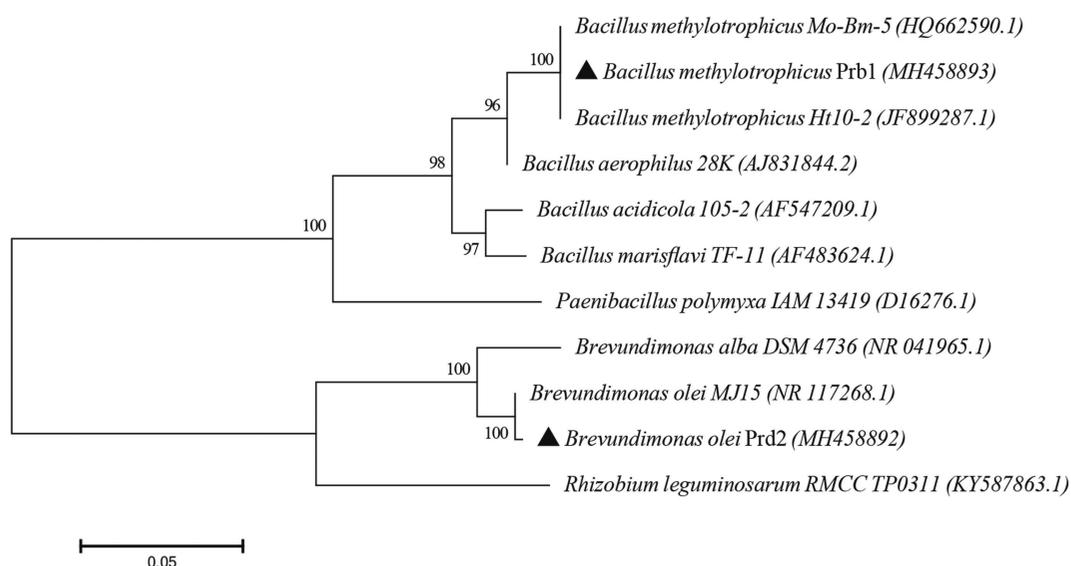


Fig. 2. Phylogenetic tree showing position of the biocontrol agents (▲) used in this study. The 16S rDNA sequences were aligned with the ClustalW and the tree was constructed with the maximum likelihood method based on the Tamura-Nei model integrated in the MEGA7 software. The GenBank accession numbers of the DNA sequences are shown in parentheses.

Table 5. Effect of concentration of ferric chloride on the mycelial growth (mm) of *Fusarium oxysporum*

Bacterial isolates	Different concentration of ferric ion			
	Mycelial growth (0 μ M)	Mycelial growth (50 μ M)	Mycelial growth (70 μ M)	Mycelial growth (100 μ M)
Prb1	9.66 ^b	25.33 ^{ab}	24.00 ^a	10.66 ^b
Prd2	10.33 ^b	10.66 ^c	7.66 ^b	11.33 ^b
Control	23.33 ^a	26.00 ^a	26.66 ^a	27.66 ^a

Values are the means of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level.

Table 6. Effect of bacterial isolates on the seed germination of two cultivars (Pusa Ruby and Ratan) of tomato

bacterial isolate	Seed germination (%)	
	Pusa Ruby	Ratan
Prd2	81 ^b	79 ^a
Prb1	84 ^b	85 ^a
Control	96 ^a	78 ^a

Values are the mean of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level.

Germination Percentage (GP) and seedling vigor. Germination of tomato seeds recorded in germination test varied among the cultivars (Table 6). Bacterization of tomato seeds (cv. Ratan), there was no significant difference observed between treatments. Whereas, tomato seeds (cv. Pusa Ruby), bacterized seeds showed significant difference over the control and the two isolates have the same effect. There was an improvement in seedling vigor upon addition of bacterial isolates to tomato seeds. Under laboratory

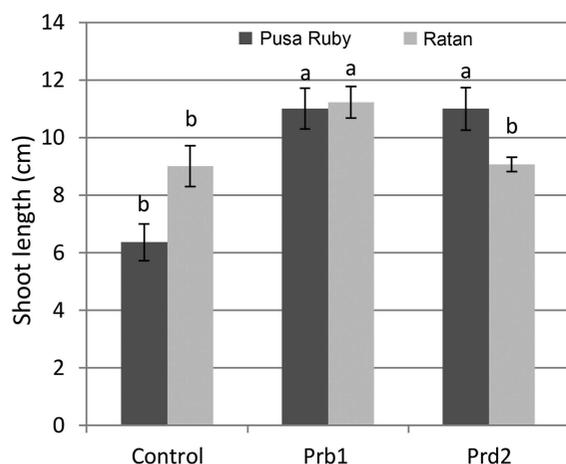
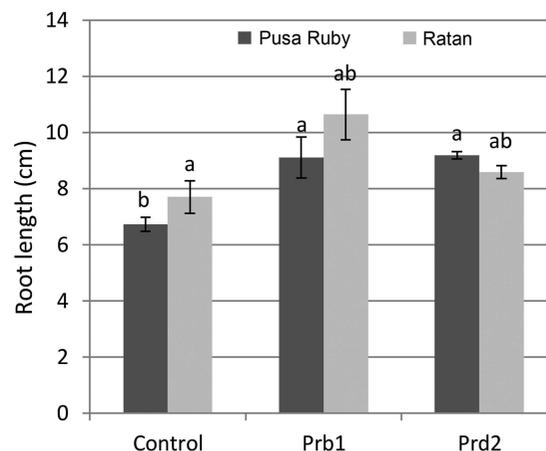
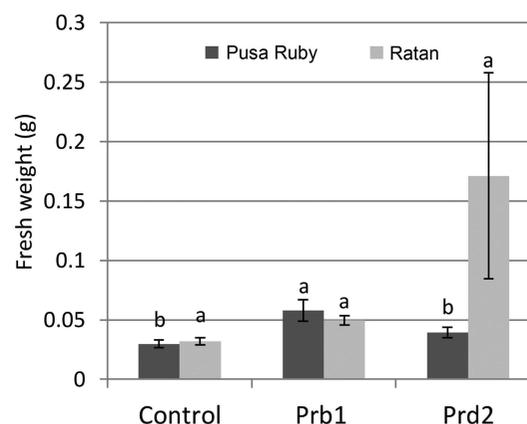
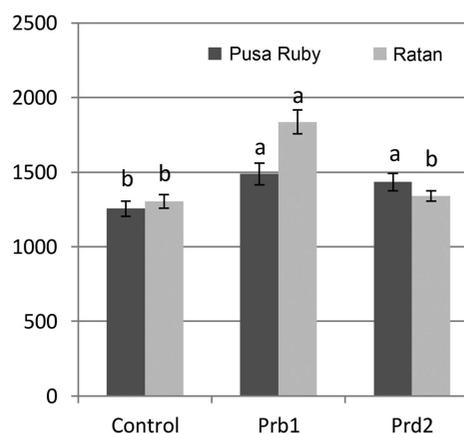
**Fig. 3.** The effect of bacterization of tomato seeds with two bio-control agents on shoot length of tomato seedlings. Data are mean of three replications. Bars indicate standard error of the mean.**Fig. 4.** The effect of bacterization of tomato seeds with two bio-control agents on root length of tomato seedlings. Data are mean of three replications. Bars indicate error of the mean.**Fig. 5.** The effect of bacterization of tomato seeds with two bio-control agents on the fresh weight of tomato seedlings. Data are mean of three replications. Bars indicate standard error of the mean.**Fig. 6.** The effect of bacterization of tomato seeds with two bio-control agents on vigor index of tomato seedlings. Data are mean of three replications. Bars indicate standard error of the mean.

Table 7. The average disease incidence and biocontrol efficacy in controlling Fusarium wilt of treated tomato plant with rhizobacterial isolates in year 2015-16 and 2016-17

Treatments	Bacterial inocula	Disease incidence 2015-16	Biocontrol efficacy (%)	Disease incidence 2016-17	Biocontrol efficacy (%)	Biocontrol efficacy (Mean of 2 years)
Pusa Ruby	Un-inoculated (Control inoculated with <i>F. oxysporum</i> f. sp. <i>lycpeorsici</i>)	41.66 ± 2.30 ^a		41.66 ± 0.58 ^a		
	Prd2	20.83 ± 3.17 ^c	50	25 ± 4.04 ^b	39.99	44.99
	Prb1	29.16 ± 0.58 ^b	30	33.33 ± 2.51 ^{ab}	19.99	24.99
Ratan	Un-inoculated (Control inoculated with <i>F. oxysporum</i> f. sp. <i>lycpeorsici</i>)	42.33 ± 4.16 ^a		58.33 ± 3.05 ^a		
	Prd2	24.99 ± 3.05 ^b	40.97	33.33 ± 6.65 ^{bc}	42.85	41.91
	Prb1	33.33 ± 2.64 ^{ab}	21.26	25 ± 3.00 ^c	57.14	39.20

Values are the means (± SEM) of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level according to Duncan Multiple Range Test.

conditions, all the isolates showed higher mean root length, higher mean shoot length, and vigor index with respect to control (Fig. 3-6). The isolate *B. methylotrophicus* Prb1 caused the maximum increase (11.22 cm) of shoot length in the Ratan cultivar, while in Pusa Ruby the two isolates have the same effect in shoot length (11 cm). Addition of the isolate *B. olei* Prd2 recorded the maximum increase of root length (9.18 cm) in Pusa Ruby, while addition of the isolate Prb1 recorded the maximum increase of root length (10.64 cm) in Ratan cultivar. Among the isolates, the isolate *B. methylotrophicus* Prb1 recorded the highest fresh weight in Pusa Ruby, while addition of the isolate *B. olei* Prd2 recorded the highest fresh weight in Ratan cultivar. Highest vigor index was observed in both the cultivars by

the addition of the isolate Prb1.

Biocontrol potential of selected bacterial isolates. In this study, disease incidence was reduced to different levels depending on the bacterial isolates. Tomato plants treated with the bacterial isolates looked healthy and showed a lower incidence of wilt disease in both of the growing seasons (Table 7). Treatment of tomato cultivar Pusa ruby with the isolate *B. olei* Prd2 reduced the disease incidence by 50% in 2015-16 and 39.99% in 2016-17 cropping seasons. Whereas treatment of tomato cultivar Ratan with the same isolate (Prd2) reduced the disease incidence by 40.97% in 2015-16 and 42.85% in 2016-17 cropping seasons.

Using *B. methylotrophicus* Prb1 and *B. olei* Prd2 isolates,

Table 8. Effect of bacterial isolates on growth parameters of tomato (cv. Pusa Ruby)

Growth parameters	Bacterial inoculums (2015-16)			Bacterial inoculums (2016-17)		
	Uninoculated	Prd2	Prb1	Uninoculated	Prd2	Prb1
Height (cm)	52 ± 2.00 ^b	66 ± 7.57 ^{ab}	75 ± 2.51 ^a	53 ± 1.52 ^b	62 ± 1.52 ^{ab}	69 ± 4.61 ^a
Leaf area (cm ²)	150 ± 3.93 ^b	234 ± 5.29 ^a	163.8 ± 4.10 ^b	396 ± 5.34 ^a	336 ± 5.95 ^a	368 ± 6.06 ^a
Leaves no./plant	44 ± 3.05 ^c	73 ± 4.50 ^a	59 ± 2.64 ^b	20 ± 1.15 ^b	18 ± 2.64 ^b	30 ± 1.15 ^a
Root length (cm)	11.1 ± 2.08 ^b	24.5 ± 1.15 ^a	22.2 ± 2.00 ^a	15 ± 2.30 ^a	12.6 ± 2.51 ^a	16 ± 2.51 ^a
Root fresh weight (g/plant)	8.25 ± 0.58 ^{ab}	12.08 ± 2.88 ^a	4.10 ± 1.00 ^c	3.59 ± 0.58 ^c	9.95 ± 1.15 ^b	22.82 ± 0.58 ^a
Shoot fresh weight (g/plant)	37.45 ± 3.46 ^b	107.21 ± 5.77 ^a	117.10 ± 4.72 ^a	61.25 ± 4.01 ^a	59.02 ± 2.08 ^a	59.15 ± 6.35 ^a
Root dry weight (g/plant)	4.23 ± 0.58 ^a	3.05 ± 1.00 ^a	1.16 ± 0.58 ^a	2.6 ± 0.58 ^b	7.81 ± 0.58 ^b	17.41 ± 2.64 ^a
Shoot dry weight (g/plant)	6.09 ± 1.00 ^b	15.68 ± 1.52 ^a	18.01 ± 0.58 ^a	31.25 ± 4.93 ^a	18.56 ± 5.29 ^a	15.38 ± 4.04 ^a
Chlorophyll a (mg/g)	1.397 ± 1.00 ^a	1.550 ± 0.577 ^a	1.899 ± 0.00 ^a	1.07 ± 0.577 ^a	1.258 ± 0.58 ^a	1.661 ± 1.00 ^a
Chlorophyll b (mg/g)	0.885 ± 0.00 ^a	0.906 ± 0.43 ^a	1.074 ± 1.00 ^a	0.579 ± 0.062 ^a	0.608 ± 0.58 ^a	0.832 ± 0.00 ^a
Total chlorophyll (mg/g)	1.971 ± 0.58 ^a	2.126 ± 1.52 ^a	2.541 ± 0.58 ^a	1.215 ± 0.58 ^a	1.572 ± 0.58 ^a	1.909 ± 1.00 ^a
Carotenoids (mg/g)	5.572 ± 2.08 ^a	5.572 ± 1.15 ^a	7.494 ± 1.52 ^a	4.061 ± 1.15 ^{ab}	4.942 ± 0.58 ^{ab}	6.690 ± 1.15 ^a

Values are the means (± SE) of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level according to Duncan Multiple Range Test.

Table 9. Effect of bacterial isolates on growth parameters of tomato (cv. Ratan)

Growth parameters	Bacterial inoculums (2015-16)			Bacterial inoculums (2016-17)		
	Uninoculated	Prd2	Prb1	Uninoculated	Prd2	Prb1
Height (cm)	61 ± 4.04 ^b	83 ± 2.51 ^a	62 ± 5.29 ^b	60 ± 1.73 ^{bc}	64 ± 3.05 ^{ab}	56 ± 2.64 ^c
Leaf area (cm ²)	215 ± 8.54 ^b	384 ± 5.68 ^a	222 ± 2.15 ^b	486 ± 7.81 ^a	450 ± 5.13 ^a	415 ± 8.80 ^a
Leaves no./plant	37 ± 4.16 ^a	37 ± 8.18 ^a	45 ± 0.57 ^a	29 ± 1.52 ^a	13 ± 2.00 ^b	28 ± 4.35 ^a
Root length (cm)	20 ± 1.00 ^a	20.2 ± 3.05 ^a	20 ± 1.52 ^a	14.9 ± 2.51 ^a	11 ± 2.51 ^a	14 ± 1.73 ^a
Root fresh weight (g/plant)	3.25 ± 0.58 ^c	16.99 ± 1.00 ^a	3.95 ± 0.58 ^b	17.5 ± 2.64 ^a	9.6 ± 1.52 ^b	6.59 ± 1.15 ^b
Shoot fresh weight (g/plant)	40.4 ± 1.70 ^a	48.10 ± 4.93 ^a	42.47 ± 7.81 ^a	52.32 ± 5.56 ^a	62.63 ± 4.04 ^a	65.46 ± 6.80 ^a
Root dry weight (g/plant)	1.43 ± 0.58 ^b	8.74 ± 1.52 ^a	2.39 ± 1.73 ^b	8.25 ± 2.00 ^a	6.09 ± 1.52 ^a	4.4 ± 1.15 ^a
Shoot dry weight (g/plant)	7.042 ± 1.15 ^a	15.71 ± 3.51 ^a	8.97 ± 2.64 ^a	9.71 ± 1.73 ^a	28.89 ± 9.01 ^a	22.54 ± 2.08 ^a
Chlorophyll a (mg/g)	1.211 ± 1.73 ^a	1.670 ± 0.58 ^a	1.745 ± 0.58 ^a	1.366 ± 0.58 ^a	1.366 ± 1.00 ^a	0.832 ± 0.00 ^a
Chlorophyll b (mg/g)	0.737 ± 0.00 ^a	0.959 ± 0.13 ^a	1.022 ± 1.00 ^a	0.652 ± 0.00 ^a	0.366 ± 0.170 ^a	0.452 ± 0.00 ^a
Total chlorophyll (mg/g)	1.676 ± 0.58 ^a	2.226 ± 1.15 ^a	2.373 ± 1.52 ^a	1.698 ± 0.58 ^a	0.925 ± 1.00 ^a	1.143 ± 0.58 ^a
Carotenoids (mg/g)	5.044 ± 1.15 ^a	6.604 ± 1.52 ^a	7.060 ± 0.577 ^a	5.662 ± 1.00 ^a	2.937 ± 1.15 ^{ab}	3.931 ± 0.58 ^{ab}

Values are the means (± SEM) of three replications. Means in a column with similar letter(s) are not significantly different at 0.05 level.

growth promotion assays were performed in two successive years. The magnitude of growth promotion varied among the bacterial isolates and tomato cultivars. Results of this experiment showed (Table 8, 9) that bacterial isolates have stimulated plant growth under pot conditions. Highest biomass increase was recorded in treatment with *B. methylotrophicus* Prb1 isolate. The effect of bacterial inoculation showed that inoculation of bacterial isolates slightly increased chlorophyll and carotenoid contents.

Discussion

The agro-ecological system and constituents of plant root exudates determine the type and density of microbial population in a given crop production system (Van Overbeek et al., 1997). Beneduzi et al. (2008) also stated that rhizospheric microbial community is determined by both soil characteristics and plant species. So, the present study was conducted to explore the potentiality of bacteria isolated from the rhizosphere soil of different agro-ecological zones (AEZs) of Bangladesh for biocontrol of *Fusarium* wilt of tomato as well as plant growth promotion.

Out of 24 randomly selected isolates, 22 (91.66%) showed different level of inhibitory activity against *F. oxysporum*. In dual cultures all bacterial isolates showed variations in inhibition of mycelial growth of *F. oxysporum*. Several workers also reported similar effects against numerous fungi including *Fusarium* species (Khan and Zaidi, 2002; Sivamani and Gnanamanickam, 1988). In the present study, variable antifungal activity of different isolates could be attributed to the quantity and effectiveness of the antimicrobials produced by the isolates.

The selected two biocontrol isolates were identified by morphological, cultural, biochemical and molecular characteristics. The isolates Prb1 and Prd2 were identified as *B. methylotrophicus* and *B. olei*, respectively. Although the presence of *B. olei* has been reported from various region of the world (Fan et al., 2016; Lee et al., 2010), no literature on the presence of *B. olei* in Bangladesh soil exists. Hence, this is the first report of *B. olei* from Bangladesh.

In present study it was found that the selected bacterial isolates showed antagonist activity against *F. oxysporum* along with one or more lytic enzyme production. The addition of FeCl₃ to Kings B at 50 and 70 µM eliminated the inhibitory effect of siderophores produced by bacterial isolates against *F. oxysporum*. This indicated siderophore mediation along with antifungal metabolites. Reduction of antagonism by the addition of iron indicated that siderophores and other defensive metabolites were inhibitory to fungal growth and showed a cumulative effect of different defensive mechanisms (Bultrey and Gheysen, 2000). Suryakala et al. (2004) reported that siderophores exerted maximum impact on *F. oxysporum* than on *Alternaria* sp. and *Colletotrichum capsici*. The selected two rhizobacterial isolates were tested for their cyanogenic activity and the result revealed that only *B. olei* Prd2 isolate produced HCN. Hydrogen cyanide production by rhizospheric bacteria is beneficial from the biocontrol point of view. This volatile metabolite is thought to play a major role in biological control of some soil borne diseases (Siddiqui et al., 2006). Among the isolates, only isolate *B. olei* Prd2 was able to grow in the Norris N-free agar medium indicating that this isolate is probably able to utilize the atmospheric nitrogen. Kumar and Gera (2014) reported that *Brevundimonas* sp.

isolated from sugarcane rhizospheric soil showed nitrogen-fixing potential. Gulati et al. (2011) also reported nitrogenase activity by acetylene reduction assay of the strains of *B. bullata*. Both the isolates (Prb1 and Prd2) showed positive for root colonization. Results of the study showed that none of these two isolates inhibited *B. subtilis*, *T. harzianum* and *T. reesei*. Study of these interactions is extremely important as an effective means for their integration with the disease management practices (Belkar and Gade, 2012) and correlating their specific role in the host-pathogen other microorganisms interactions (Mishra et al., 2011).

In the present study, *Brevundimonas olei* and *Bacillus methylotrophicus* were able to significantly enhance all measured plant growth parameters. There was variation in germination and seed vigor index in both the cultivars with the bacterial treatments when compared to controls indicating that microbial inoculants can enhance seed vigor index. Similar effects of the rhizobacteria were also reported by other authors in tomato (Murthy et al., 2014) and pearl millet (Niranjan et al., 2003).

Disease incidence was reduced to different levels depending on the bacterial isolates and was estimated up to 57.14 compared to control on the tomato plants. The use of bioagents was reported quite effective to control Fusarium wilt disease in tomato (Freeman et al., 2002). In the present study, *B. methylotrophicus* Prb1 showed highest *in vitro* antagonistic activity but in the pot culture isolate *B. olei* Prd2 showed slightly higher disease inhibition than Prb1. This may be due to increased fitness of the isolate *B. olei* Prd2 to the biotic and abiotic factors of the experimental field. Wei et al. (2017) stated that microbe-microbe interactions are very sensitive to several abiotic and biotic factors such as environmental temperature, productivity and microbial community composition, which could affect biocontrol outcomes by changing the strength of species interactions. In the experiment, application of *B. olei* reduced 44.99 and 41.91% of disease incidence of two tomato cultivar Pusa Rubi and Ratan, respectively as compared to infected untreated control. Application of *B. methylotrophicus* also reduced percentage of disease incidence of tomato plants as compared to infected untreated control. Thus, these treatments improved plant health through reducing wilt symptoms. Our results also corroborated with the findings of other researchers (Almoneafy et al., 2012; Ge et al., 2016).

The growth responses of test plant tomato to rhizobacterial treatments varied significantly with the cultivar-type and isolate type. The variations in plant growth promotion among the isolates are attributed to their individual competencies (Geetha et al., 2014). The selected isolates

significantly increased root length, shoot length and exhibited biomass increase of treated tomato plants compared to control plant. Present results were in agreement with those reported by Mezeal (2014), who reported that the growth parameters of tomato plants were increased with the addition of *B. subtilis* and *P. fluorescens* to the soil with *F. oxysporum* under field condition.

Extensive rooting was observed in bacteria-treated tomato plants compared with the control. The rooting might have contributed towards the resistance, growth and development of the plant. According to Raj et al. (2003), the possible mechanisms could be the larger and healthier root system leading to improved uptake of water and nutrients. In respect of *B. methylotrophicus* our results corroborated with the findings of Ambawade and Pathade (2015) and Ge et al. (2016). In respect of *Brevundimonas* species, similar improvement of growth parameters has been reported in wheat (Rana et al., 2011) and Bt-cotton (Kumar and Gera, 2014). Changes in plant pigments of two cultivars of tomato during tomato growth season 2015-16 and 2016-17 was also observed. The results shown that bacterial treatments increased chlorophyll and carotenoid contents in tomato plants compared to the untreated control. Similar pattern of influence was also reported in other studies (Lamsal et al., 2013). Application of rhizobacterial isolates might have affected the production of these biochemicals in plants. These plants mediated mechanism had been proposed by researchers as regulating force behind plant growth promotion by PGP bacterial strains (Silva et al., 2003).

In conclusion, among the 24 rhizobacterial isolates tested, we have found two potential indigenous soil-borne antagonists, *Brevundimonas olei* Prd2 and *Bacillus methylotrophicus* Prb1. The selected rhizobacterial isolates are capable of reducing Fusarium wilt disease under local agroclimatic conditions of Bangladesh. This study reports new native isolates of *B. olei* with potential to control not only Fusarium wilt disease but also promoted growth of tomato plant. Therefore, these bacteria could be used at the field level to control the *F. oxysporum*. However, further studies on different formulations for practically implementing such propositions will be required.

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