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CHARACTERIZATION AND EVALUATION OF *BACILLUS SIAMENSIS* ISOLATE FOR ITS GROWTH PROMOTING POTENTIAL IN TOMATO

AMANUL ISLAM*, MD. SHAHINUR KABIR, ABUL KHAIR

Jahangirnagar University, Savar, Bangladesh

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In quest of prospective rizospheric bacteria of agricultural importance, one of the isolates from bean (*Lablab niger* Medikus) was identified as *Bacillus siamensis* based on morphological, biochemical and 16S rRNA gene sequencing data. Study was carried out to evaluate growth promotion of two tomato cultivars*, in vitro* and *in vivo*. Experiments conducted for two consecutive years, following seed treatments revealed that the bacterial isolate increased plant height by 14.66–15.68%, shoot fresh weight by 34.5–65.09% and root fresh weight by 75.3–92.48% over the non-treated control. The bacterial strain showed encouraging results for plant growth promotion in pot study and hence may be useful for the growth enhancement of tomato plant.

Key words: 16S rRNA gene, *Bacillus siamensis*, growth promotion, plant growth-promoting rhizobacteria, tomato

Dependence on chemical inputs are major means of modern day agriculture management, which have created a negative impact on agro-ecosystems. There is an immediate need to minimize the use of hazardous agrochemicals by alternative ways. The utilization of plant growth promoting bacteria as bio-fertiliser offers an attractive option to replace synthetic chemical fertiliser, pesticides and supplements. Rhizobacteria, that live in the plant rhizosphere play a vital role in maintaining soil quality and upgrading plant growth and development. Various plant growth-promoting rhizobacterial (PGPR) strains such as *Bacillus*, *Pseudomonas*, *Azotobacter, Azospirillum* spp. are being used to develop organic biofertilisers. PGPR promote plant growth through nutrient recycling, nitrogen fixation, synthesis of the phytohormone, solubilization of nutrients such as P, K and Fe, and enhancing plant resistance to pests and diseases (Alan & Kiran 2018).

Commercial applications of PGPR are being investigated and researchers are able to use them successfully as inoculants from laboratory to field (Sivasakthi *et al*. 2014). There are fewer than 20 different PGPR strains which are known to be available commercially (Kloepper *et al.* 2004). Commercialization efforts have been hampered due to variable strain performance. However, effective PGPR number can be increased by isolating promising native strains, which are more suitable as bio-inoculants due to a better environmental adaptation (Sicuia *et al.* 2015). Considering the beneficial roles of PGPR in sustainable agricultural systems, it would be very economical to search and document prospective native isolates.

Tomato is an important vegetables in Bangladesh. Currently, tomato ranks first in respect of production and second in respect of area from all vegetables grown in the country (BBS 2016). To meet up local demand, Bangladesh government imported

Amanul Islam (*Corresponding author), Md. Shahinur Kabir, Abul Khair, Department of Botany, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh. E-mail: progoti17@gmail.com

28,76,613 metric tons of tomato from foreign countries in the year 2014–2015 (BBS 2016). Considering the country's limited arable areas for vegetables, studies about eco-friendly farming systems is necessary to achieve increase of production in order to keep pace with increasing population in the country. In this context of Bangladesh with emphasis in tomato crop, the present study was made to find promising native rhizospheric bacterial isolates as a plant growth promoter so that it could contribute to biofertilisation.

The goal of the work was to search prospective bacterial isolate from agricultural fields which can be used to develop as environment-friendly biofertiliser for tomato plants cultivated in different agro-ecological zones (AEZ) of Bangladesh. The specific aim of the present work was to evaluate the growth promotion potentiality of tomato plant using a bacterium isolated from bean rhizosphere.

MATERIAL AND METHODS

Sample collection and isolation of bacteria from the rhizosphere

Bean (*Lablab niger* Medikus) rhizosphere soil samples along with the roots were collected from a farmer's field located in Nobinagar, Brahmanbaria district, Bangladesh. The isolate was obtained from rhizosphere soil from old Meghna estuarine floodplain agro-ecological zone (AEZ) of Bangladesh and the soil belongs to predominantly silty, but silty clay and clay also found. Soil samples were air dried and brought to room temperature (28 ± 1°C). One gram of each of the collected soil were weighed and placed in the test tube containing 9 mL of 0.9% NaCl solution (Bahig *et al.* 2008). The suspension was serially diluted and 0.1 ml of an appropriately diluted culture was spread onto the nutrient agar (NA; containing g/l: 3 g Beef extract, 5 g Peptone, 15 g Agar) plates to isolate rhizobacteria from soil. The nutrient agar plates were incubated at 28 ± 2ºC for 24 h. After incubation of the inoculated plates, various types of bacterial colonies were developed. Bacterial colonies of different morphology were randomly selected and purified. One of the isolates was designated as Pbbb1 and selected for further study.

Characterization of the isolate Pbbb1

Morphological and biochemical tests were carried out to characterize the bacterial isolate Pbbb1 by using standard procedures (Schaad *et al.* 2001) such as Gram-staining 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. Growth parameters of bacterial isolate were studied using nutrient broth (NB) under various NaCl concentrations, varying temperatures and pH using a turbidometric method (Chookietwattana & Maneewan 2012). Growth rate (μ), doubling time (t_d) and multiplication rate (MR) of the Pbbb1 was calculated according to Painter and Marr (1968) and Stanier *et al.* (1970), respectively.

Production of extracellular hydrolytic enzymes such as protease, cellulase and amylase, were tested by growing Pbbb1 on the medium containing enzyme substrate; skim milk for protease assay (Maurhofer *et al.* 1995), carboxymethyl cellulose for cellulase assay (Shivakumar *et al.* 2013) and starch for amylase assay (Schaad *et al.* 2001). Development of halo zone surrounding the colony was considered as positive result. HCN production was determined using Bakker and Schippers (1987) method. Siderophore production was tested using method described by Naik *et al.* (2008). The ability of the bacteria to grow in N-free medium was determined following the methodology of Kumar *et al.* (2012). Isolate was streaked on sterile Norris Agar plate. The method described by Silva *et al.* (2003) was used to determine root colonization bioassay.

16SrRNA sequencing for identification of Pbbb1

Genomic DNA of Pbbb1 was isolated from the bacterial colony using Maxwell Cell Kit (Promega, USA) and Maxwell 16 automated DNA extractor following manufacturer's instructions. The purity and DNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific, U.S.A.). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primer pair 1492R (5'-GGTTACCTTGTTACGACTT-3') and 27F(5'-AGAGTTTGATCMTGGCTCAG-3'). DNA concentration of the sample was 197.2 ng/μl. Ampli-

fication reactions were performed in a total volume of 25 µl containing 1 µl (60 ng) of DNA template, 1 µl of each forward and reverse primer (10 µM), 9.5 µl water and 12.5 µl Master Mix (Promega, USA). To amplify the target region, thermal cycling was performed as follows: initial denaturation at 95°C for 4 min, followed by 30 cycles in which each cycle consists of denaturation at 95°C for 30 s, annealing at 49°C for 30 s, followed by extension at 72°C for 1 min 25 s. The final extension was done at 72°C for 5 min. These reactions were carried out using a thermocycler (Gene Atlas, Astesc, Japan). The PCR product was resolved by electrophoresis in 1% agarose (w/v) gel submerged in 1 × TAE buffer. The amplified DNA band was visualized using a transilluminator after staining with ethidium bromide. The PCR product was sequenced in both directions at 1st Base Laboratories, Malaysia. The sequence obtained was used as a query sequence to identify the bacterial isolate by using EZBioCloud (https://www.ezbiocloud.net/) identification tool (Yoon *et al*. 2017). The 16S rRNA gene sequence of the bacterial isolate was deposited to GenBank and accession number was obtained (MH458894).

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

Effect of selected bacterial isolate on plant growth parameters of tomato

Plant growth promotional efficacy of Pbbb1 isolate was evaluated in tomato. Tomato cultivars Ratan and Pusa Ruby were used in this study because of popularity of these cultivars among Bangladeshi farmers. Seed surface sterilization and water formulated bacterial inoculation was followed as described by Weller & Cook (1983). Seed samples were soaked in cell suspension of Pbbb1 isolate at concentration of approximately 1×10^8cfu/ml. Seedling vigor test was performed using the standard roll towel method (ISTA 1999). Treated and untreated seeds placed equidistantly in between two wet paper towels and the papers were rolled. Seeds were allowed to germinate at 25ºC. After 10 days of incubation seedlings were measured for (a) seed percentage germination (b) root length (c) shoot length (d) seedling weight and (e) vigor index were calculated. The vigor index of the seedling was calculated by using the following formula (ISTA 1999):

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

For a pot experiment the bacterial isolate was tested in sterilized soil. Pot experiment was performed in soil (clay 87%, loam 13% and sand <1%) of Madhupur tract AEZ. The experiment was carried out at the experimental site of Botanical Garden, Department of Botany, JU, Bangladesh (23°88' N and 90°26' E) during two successive local crop seasons (December 2015 to February 2016 and December 2016 to February 2017). For pot experiments soil was prepared and sterilized following the method described by Khalequzzaman *et al.* (2002). Bacterial inoculation was done as described earlier. Control consists of nonbacterized seed. Seeds inoculated with the isolate and without the isolate were permitted to air dry for 6 hrs before planting. Seeds were then sown in earthen pots (diameter: 31 cm, height: 31 cm).

The pots were arranged in randomized manner and three plants grown per pot. Three replications were maintained for each treatment. The tomato crop was raised for 90 days. There after the crop was uprooted. The growth parameters namely plant height, number of leaves, leaf area, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight of plants raised from treated and untreated seeds were recorded. The estimation of total chlorophyll was determined according to Arnon (1949):

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

where:

D – optical density at wavelength indicated; V – final volume of extract; W – fresh weight of leaf material used; d – length of light path in cm.

The values were expressed as mg/g of the fresh leaf weight.

Statistical analysis

Data were analyzed for significant mean differences using two-way analysis of variance (ANOVA). Means were separated using Duncan's multi-

ple range test (DMRT; $\alpha = 0.05$) using SPSS software.

RESULTS AND DISCUSSION

In this study an isolate was tested for growth promotional efficacy under different conditions during the winter crop growing season for two successive years. The present work also focused on native rhizospheric bacterial isolate. Because co-existence for many years with the natural soil, microbiota should provide native microorganisms with competitive advantages compared to exotic species (Figueroa-López *et al.* 2016).

Molecular identification of isolated bacteria

The isolate Pbbb1 obtained from the rhizosphere of bean was characterized morphologically, biochemically and subjected to 16S rRNA gene sequence analysis. The study revealed that isolate Pbbb1 is Gram positive and appeared as rod shaped (Figure 1). Colony was small, irregular, round yellowish white on NA medium. Results of biochemical characterization (Table 1) indicated that Pbbb1 was positive for oxidase test, gelatin liquefaction and arginine decarboxylase activity. The isolate could utilize lactose and xylose. Specific growth rate, doubling time and multiplication rate of the isolate in nutrient broth were 0.0099/h, 69.59 min and 0.0143/h, respectively. With respect to protease activity, siderophore production and root colonization by Pbbb1 was found positive (Table 1).

The isolated bacteria was further identified at the molecular level. The comparative 16S rRNA gene sequence-based identification using EZBioCloud bioinformatics tool revealed that the 16S rRNA gene of the isolate is 99.72% identical to that of *Bacillus siamensis* KCTC 13613 (T). The result of molecular analyses was consistent with the morphological, biochemical and physiological traits of the isolate. Results on the biochemical reactions performed in this study such as nitrate reduction, oxidase, catalase, arginine test, gelatin liquefaction, certain carbohydrate utilizations and morphological features were similar to the result reported by Almoneafy *et al*. (2012) and Ambawade & Pathade (2015). Based on the studies conducted, the bacterial isolate was identified as *Bacillus siamensis*.

Bacillus spp. are well known rhizosphere residents of many crops. The occurrence and isolation of *Bacillus* species from the rhizosphere of differ-

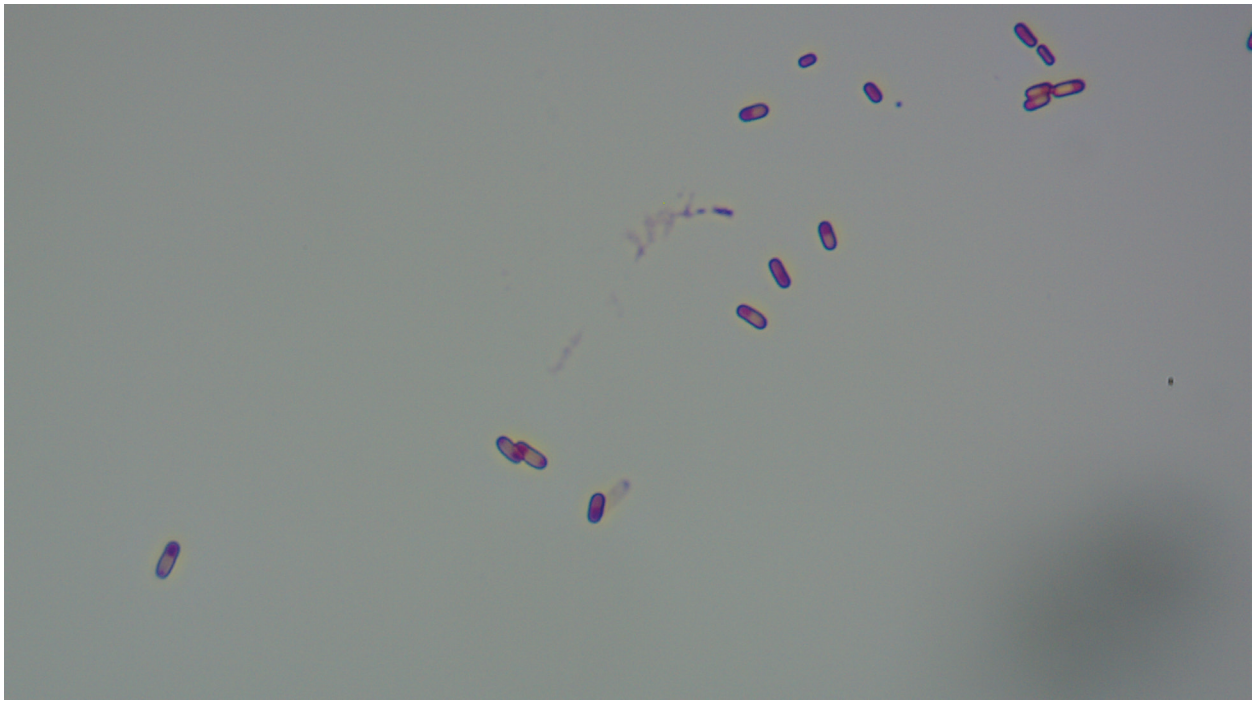

Figure 1. Microscopic image showing cells of isolate Pbbb1

ent plants have been reported by different authors (Cabra *et al.* 2017; Almoneafy *et al.* 2012). This is probably the first report of isolation of *B. siamensis* from Bean rhizospheric soil of Bangladesh. However, information on *B. siamensis* as a growth promoting microorganism is scanty. Being considered as safe microorganism and holding the remarkable abilities of synthesizing a vast array of beneficial substances (Stein 2005) we targeted *Bacillus* as the chosen bacterial species.

Effect on the growth of tomato seedlings

In the present study, the ability of *B. siamensis* for promotion of tomato plant growth was investigated *in vitro* using the standard roll towel method. The bacterial seed treatment did not significantly decrease or increase the germination percentage. Under laboratory conditions, isolate was able to promote seedling growth and enhance vigor index. The effect was more pronounced in cv. Ratan (Table 2).

T a b l e 1

Biochemical, physiological characterization and beneficial traits of isolate Pbbb1

Physiochemical indices	Characteristics
Physiological characteristics:	
Growth in NaCl at concentration (1–5%)	+
Growth at different temperature (20–41ºC)	+
Growth at different pH (6–8)	+
Growth on nitrogen free medium	–
Biochemical tests:	
Gram reaction	+
Oxidase test	+
Catalase test	–
Gelatin liquefaction	+
Nitrate reduction	–
Argininedecarboxylase activity	+
Levan formation	–
Urease test	–
Carbon sources utilization:	
Glucose, Maltose, Mannitol	–
Lactose, Xylose	+
Secondary metabolites production and beneficial traits:	
Starch hydrolysis test	–
Protease activity	+
Cellulase activity	–
HCN production	–
Siderophore production	+
Root colonization test	+

‘+’indicates positive; ‘–’indicates negative

The mean shoot length was increased in bacteria treated cv. Pusa Ruby (10.3 cm), cv. Ratan (9.92 cm) and lowest in control. The mean root length was increased in treated cv. Ratan (11.02 cm), cv. Pusa Ruby (7.82 cm), and lowest in control. Fresh weight also increased in bacterial treated germinating seedlings. Inoculation enhanced the vigor index when compared to control in cultivar Ratan. However, no significative difference was observed between treatments in the cultivar Pusa Ruby. Similar pattern of

T a b l e 2

The effect of bacterization of tomato seeds with Pbbb1 on seedling vigor of tomato seedling under *in vitro* conditions (as described by ISTA 1999)

Treatments		Shoot length [cm]	Root length [m]	Fresh weight [g]	Vigor index
Pusa Ruby	Pbbb1	10.3±0.33^{a}	7.82±0.20^{a}	0.04±0.00^{ab}	1195.92±29.47^{a}
	Control	6.36±0.64^{b}	6.72±0.25^{a}	0.02±0.00^{b}	1255.68±51.10^{a}
Ratan	Pbbb1	9.92±0.40^{ab}	11.02±1.54^{a}	0.04±0.00^{ab}	1633.32±134.70^{a}
	Control	9.00±0.70^{b}	7.70±0.58^{b}	0.03±0.00^{b}	1302.6±45.48^{b}

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

T a b l e 3

Effect of bacterial isolate on growth parameters of tomato plants under pot conditions in cv. Pusa Ruby

Bacterial Treatment		Height [cm]	Leaf area [cm^2]	Leaves no./plant	Root length [cm]	Root fresh weight [g/plant]	Shoot fresh weight [g/plant]	Root dry weight [g/plant]	Shoot dry weight [g/plant]	Total chlorophyll [mg/g]
2015−16	Uninoculated	52±2.00^{b}	150±3.93^{b}	44±3.05^{b}	11.1±2.08^{b}	8.25±0.57^{ab}	37.45±3.46^{b}	3.59±1.52^{a}	6.09±1.00^{b}	1.97±0.57^{a}
	Pbbb1	62±6.42^{ab}	374±4.16^{a}	68±2.64a^{a}	22.1±0.57^{a}	9.70±1.15^{ab}	60.36±1.41^{a}	4.23±0.57^{a}	17.93±1.00^{a}	1.91±0.57^{a}
2016−17	Uninoculated	53±1.52^{b}	396±5.34^{a}	20±1.15^{b}	15±2.30^{a}	3.59±0.57^{b}	61.25±4.01^{b}	2.6±0.57^{a}	28.25±4.93^{a}	1.21±0.57^{a}
	Pbbb1	60±2.00^{ab}	450±3.83^{a}	41±3.21^{a}	14.6±2.08^{a}	9.60±2.88^{a}	103.52±6.92^{a}	6.20±2.00^{a}	37.5±10.40^{a}	0.69±0.00^{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

T a b l e 4

Effect of bacterial isolate on growth parameters of tomato plants under pot conditions in cv. Ratan

Bacterial Treatment		Height [cm]	Leaf area [cm^2]	Leaves no./plant	Root length [cm]	Root fresh weight [g/plant]	Shoot fresh weight [g/plant]	Root dry weight [g/plant]	Shoot dry weight [g/plant]	Total chlorophyll [mg/g]
2015−16	Uninoculated	61±4.04^{b}	215±8.54^{b}	37±4.16^{b}	20±1.00^{a}	3.25±0.57^{b}	40.4±1.70^{a}	1.43±0.57^{a}	7.04±1.15^{a}	1.67±0.57^{a}
	Pbbb1	72±3.46^{ab}	340±1.10^{a}	80±2.88^{a}	21.1±6.00^{a}	6.38±1.89^{a}	62.10±2.51^{a}	3.94±2.00^{a}	12.91±2.08^{a}	1.29±0.00^{a}
2016−17	Uninoculated	60±1.73^{b}	486±7.81^{a}	29±1.52^{a}	14.9±2.51^{a}	11.34±2.51^{ab}	52.32±5.56^{a}	8.25±2.00^{a}	9.71±1.73^{b}	1.69±0.57^{a}
	Pbbb1	68±0.57^{a}	504±9.39^{a}	35±4.16^{a}	13.9±2.64^{a}	17.5±2.64^{a}	70.62±9.00^{a}	7.93±3.00^{a}	18.71±7.00^{a}	0.71±0.71^{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

improved seedling growth of tomato plants by *Bacillus* was earlier reported by Cabra *et al*. (2017). Present results agreed with Alam *et al.* (2003), who reported the cultivar variation in the stimulation of rice growth inoculated with plant growth-promoting bacteria. Different species or cultivars may produce different types of root exudates, which may support the activity of the inoculums or serve as substrate for the formation of biologically active substances by the inoculums (Khalid *et al.* 2004).

Growth promotion potentiality of the bacterial isolate under pot culture conditions

The data presented in the Table 3 and 4 showed that in pot experiment *B. siamensis* inoculated tomato seeds influenced plant height, number of leaves, leaf area, root length, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight of both tomato cultivars. However, there were differences in growth promotion between the two cultivars. Inoculation increased plant height by 14.66% in cv. Pusa Ruby and 15.68% in cv. Ratan over the control plants. Similarly root length increased by 49.54% in cultivar Pusa Ruby and 2.5% in cv. Ratan relative to the control. In case of Pusa Ruby shoot fresh weight increased by 65.09% and 34.5% in cv. Ratan over the control. As compared to control root fresh weight increment recorded 92.48% in cv. Pusa Ruby and 75.31% in cv. Ratan as compared to the control. Dry matter content also increased in both the cultivars. Increased number of leaves, leaf area was also observed in comparison to the control. No significant differences were seen between the treated and untreated control in respect of chlorophyll content.

A recent study (Ambawade & Pathade 2018) indicated that *B. siamensis* isolated from wild banana rhizosphere is capable to produce Indole acetic acid (IAA) which is one of the most significant physiologically active phytohormone. In general, the bacterial isolate significantly increased root length, shoot length and exhibited biomass increment in the treated tomato cultivars, which is in concordant with the results obtained in earlier studies conducted with the *Bacillus* strains (Almoneafy *et al.* 2014; Sana *et al.* 2014).

The increase in shoot and root growth, number of leaves, leaf area, dry matter production in response to *B. siamensis* may be attributed to the synthesis of phytohormones, increased uptake and availability of nutrients, biocontrol abilities, and triggering plant

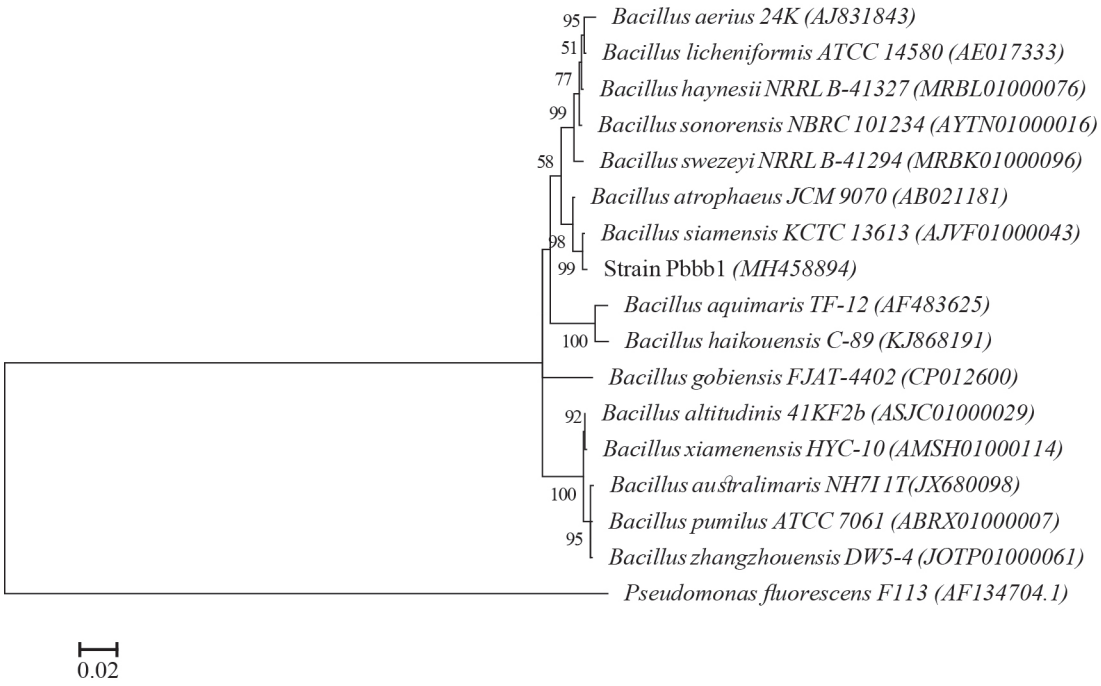

Figure 2. Phylogenetic tree showing position of the bacterial isolate used in this study. The 16S rDNA sequences were aligned with the ClustalW and the tree was constructed with the maximum likelihood method based on the Tamura-Nei model integrated in the MEGA7 software. The GenBank accession numbers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

resistance (Figueiredo *et al.* 2016). Application of *Bacillus* species to seeds or roots has been shown to cause alteration in the composition of rhizosphere microflora leading to increase in growth of plants (Vessey & Buss 2002).

CONCLUSIONS

The results of this study demonstrated that the bacterial strain *B. siamensis* Pbbb1 has the potential to positively influence the growth and development of tomato plant. The isolate was obtained from a single AEZ of the country but it may be useful in tomato crop fields of other AEZs. Further work on the response of other crops to this bacterium need to be evaluated.

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