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A Highly Salt-Tolerant Bacterium *Brevibacterium sediminis* Promotes the Growth of Rice (*Oryza sativa* L.) Seedlings

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Abstract: Soil salinity has emerged as a serious issue for food security due to global climate change. It is estimated that currently about 62 million hectares or 20 percent of the world's irrigated land is affected by salinity. Salinity is a serious problem in the coastal areas of Bangladesh. Isolation of salt-tolerant plant growth-promoting bacteria (PGPB) and applying them as bioinoculants in crop plants are considered promising and effective biotechnological approaches to combat soil salinity. This study aimed to screen salt-tolerant PGPB from the root, leaf, and rhizospheric soils of rice plants collected from salt-affected coastal areas including Chattogram, Noakhali, Lakshmipur, and Cox's Bazar districts of Bangladesh and evaluated their performances on the seedling growth of rice. Out of forty-one salinity-tolerant bacterial isolates screened, *Brevibacterium sediminis* showed salinity tolerance up to 12% NaCl (*w*/*v*). In vitro bioassay revealed that *B. sediminis* promoted the seedling growth of rice cv. BRRI dhan29 (salinity susceptible) and BINAdhan-10 (salinity tolerant), and the growth-promoting effects were higher in BINAdhan-10. This study for the first time identified *B. sediminis* strain IBGE3C as a salt-tolerant PGPB from a widely cultivated rice variety, BRRI dhan28 in the Lakshmipur district of Bangladesh. Our results suggest that salt-tolerant PGPB isolated from the root, leaf, and rhizospheric soil of rice plants could be used as a low cost and environmentally friendly option for overcoming the detrimental effects of salt stress on rice plants in the southern coastal regions of Bangladesh. However, further studies are needed for assessing the efficacy of *B. sediminis* on enhancement of salinity tolerance, and growth and yield of rice under salinity stressed conditions.

Keywords: soil salinity; salinity stress; plant growth promotion; microorganism; crop production; climate change

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1. Introduction

Many crop plants are sensitive to the high salt concentration in soils that inhibits their growth and productivity [1]. Salinity is a serious environmental hazard for plants and it significantly affects the agricultural system throughout the world [2]. Moreover, the situation is getting worse day by day because of the global tidal surge in coastal regions due to global climate change [3]. Soil salinity is a prominent threat to food security [4], mostly in the developing countries of South and Southeast Asia where the major crops are rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) [5]. The coastal regions are occupied 20% of the total land along with covering 30% of the total cultivable land in Bangladesh [6]. However, scientists are working to cope with the problems of salinity and their strategies include resource management and developing better salt-tolerant cultivars

including transgenics [7]. Since all of these strategies are lengthy and cost-intensive [8–10], there is a need for research on developing a novel, sustainable, and cost-effective approach. In this regard, the role of microorganisms, especially plant growth-promoting bacteria (PGPB), is a very important for enhancing salinity stress tolerance in crop plants [11].

Water and soil resources are the main aspects of agricultural practices and their impacts are vital [12], but the 21st century starts with the problems of the global water scarcity and salinization of soil and water [13]. The current world is struggling to establish sustainable development in agriculture because of population increase,, thereby decreasing cultivable land [14]. When the electrical conductivity (EC) of the saturation extract (ECe) in the root zone increases to 4 dS m^{-1} (about 40 mM NaCl) at 25 °C and has an exchangeable sodium of 15% or more, then the soil is called saline soil [15]. It was estimated that more than half of the total cultivable land would be salinity-affected by the year 2050 [13]. In addition, many reasons, such as irrigation with saline water and poor cultural practices, are increasing the amount of salinized soil at a significant rate every year [16]. Along with decreasing yield, salt stress also alters the quality of crops [17]. Salinity strikes plant in various ways, including abnormalities in flowering and fruiting patterns [18], and delayed formation of roots and shoots [19]. Although plants have self-defense mechanisms [20], they cannot cope with severe salinity [21]. Therefore, additional approaches such as inoculating plants with PGPB offer a great advantage to combat salt stress [21]. The PGPB has remarkable impacts to ensure normal growth and development of plants under salinity [22]. A good number of bacterial strains, such as *Bacillus* spp., *Pseudomonas* spp., *Frankia* spp., and *Rhizobia* spp., have been identified that are capable of aiding plants to tolerate various environmental stresses including salinity [11,22,23]. In addition, the magnificent benefits of the PGPB have developed a large trading platform; the commercialization of PGPB has been reported by Vessey [24] and Lucy et al. [25]. Rice is the most extensively cultivated and staple food crop in Bangladesh [26–28]. The salinity of soils in the southern coastal regions in the country is a big problem for rice cultivation. Due to climate change, the problem is increasing day by day.

Salinity-tolerant bacteria associated with rice plants can be exploited as natural bioagents for the enhancement of growth and yield of rice in salinity-affected areas. We hypothesized that the isolation and characterization of potential salt-tolerant PGPB strains could be utilized as climate-smart and cost-effective agricultural technologies in the salinity-prone coastal areas of Bangladesh. Therefore, the present study aimed to: (i) isolate and screen potential salt-tolerant bacterial isolates from rice plants cultivated in the soils of saline-prone regions of Bangladesh; (ii) identify and characterize salinity tolerant rice-associated bacteria having plant growth-promoting features using 16S rRNA gene sequencing; and (iii) evaluate some selected salinity tolerant rice-associated bacteria on seedling growth of rice.

2. Results

The results of the current study are divided into three sections—(i) collection of rice plant samples (Figures 1 and 2) from saline soils, and isolation and screening of the salinity tolerant bacteria from rice plants grown in the saline soils; (ii) performance evaluation of the screened salt-tolerant bacterial isolates on seedling growth of rice cv. BRRI dhan29 (a salinity susceptible variety) and BINAdhan-10 (a salinity tolerant variety); and (iii) identification of the best salt tolerant PGPB using 16S rRNA gene sequencing.

2.1. Isolation and Screening of Salinity-Tolerant Bacteria

To isolate and screen the effective salinity-tolerant bacteria, isolated bacteria from the rice plants were cultured in NBA medium containing the varying concentrations: 2%, 4%, 6%, 8%, 10%, and 12% (*w*/*v*) of NaCl, and the growth of the bacteria was recorded. Forty-one bacterial isolates including 8 isolates (BTNSo1–BTNSo8) from rice plants grown in the saline soils of Noakhali, 16 isolates (BTCoR1-BTCoR4, BTCoL1-BTCoL6, BTCoSo1-BTCoSo6) from Cox's Bazar, 6 isolates (BTChL1, BTChL2, BTChR1, BTChR2, BTChSo1,

BTChSo2) from Chattogram, and 11 isolates (BTLSo1-BTLSo10 and IBGE3C) from the Lakshmipur district were tested (Table 1).

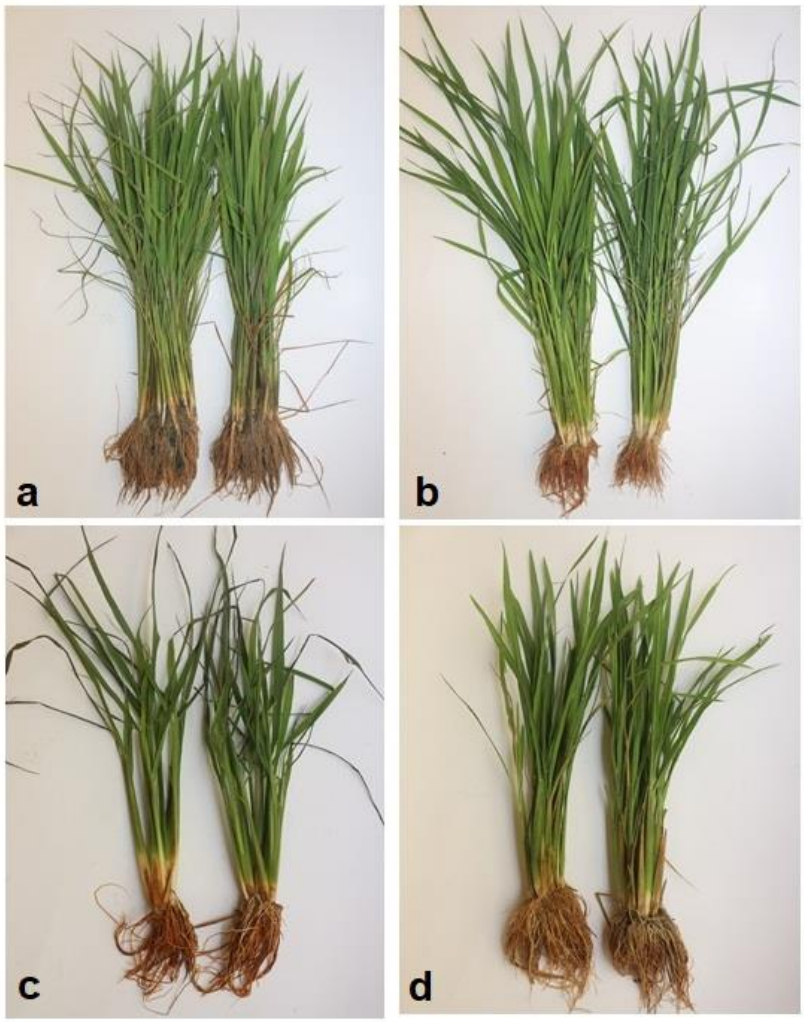

Figure 1. Collected rice plant samples from four different districts of Bangladesh for isolation of salinity tolerant PGPB. Images (**a**–**d**) of rice plants were collected from the farmers fields in Chattogram, Cox's Bazar, Lakshmipur, and Noakhali districts of Bangladesh. The age of the collected plants ranged from 35–50 days.

Figure 2. Rice plant sample collection sites (black bullets) in Bangladesh.

Table 1. Growth of isolated bacteria from different districts in the NBA medium containing varying concentrations of NaCl (*w*/*v*).

Name of Isolates	% NaCl Concentration (*w*/*v*)						
	Control	2%	4%	6%	8%	10%	12%
BTNSo1	+++	+++	+++	++	NG	NG	NG
BTNSo2	+++	++	+++	+++	NG	NG	NG
BTNSo3	+++	+++	+++	+++	NG	NG	NG
BTNSo4	+++	+	+	++	NG	NG	NG
BTNSo5	+++	+++	+++	+++	++	++	+
BTNSo6	+++	+++	+++	+++	NG	NG	NG
BTNSo7	+++	+++	+++	+++	NG	NG	NG
BTNSo8	+++	++	++	+	NG	NG	NG
BTCoR1	+++	+++	+	+	NG	NG	NG
BTCoR2	+++	+++	+++	++	+++	++	++
BTCoR3	+++	++	+++	+++	+++	++	+
BTCoR4	+++	+++	+++	+++	+	NG	NG
BTCoL1	+++	+++	+++	+++	+++	+	NG
BTCoL2	+++	+++	+++	+++	+++	+	NG
BTCoL3	+++	+++	+++	++	++	+	NG
BTCoL4	+++	+++	++	+	NG	NG	NG
BTCoL5	+++	+++	++	++	+++	+	NG
BTCoL6	+++	+++	+++	+	++	+	NG
BTCoSo1	+++	+++	+++	+	+	NG	NG
BTCoSo2	+++	+++	+++	+++	+++	+++	++
BTCoSo3	+++	+++	+++	+++	NG	NG	NG
BTCoSo4	+++	+++	+++	+	+	NG	NG
BTCoSo5	+++	+++	+++	+	NG	NG	NG
BTCoSo6	+++	+++	+++	+	NG	NG	NG
BTChL1	+++	+++	+++	+++	+++	+	NG
BTChL2	+++	+++	+++	+++	+++	+	NG
BTChR1	+++	+++	++	+	+	NG	NG
BTChR2	+++	+++	+++	++	++	+	NG
BTChSo1	+++	+++	+++	+++	NG	NG	NG
BTChSo2	+++	+++	+++	++	NG	NG	NG
BTLSo1	+++	+++	++	+	NG	NG	NG
BTLSo2	+++	+++	+++	+	NG	NG	NG
BTLSo3	+++	+++	+++	++	NG	NG	NG
BTLSo4	+++	+++	+++	+++	NG	NG	NG
BTLSo5	+++	+++	+++	++	NG	NG	NG
BTLSo6	+++	+++	+++	++	NG	NG	NG
BTLSo7	+++	+++	+++	++	+	NG	NG
BTLSo8	+++	++	+++	+++	+	NG	NG
BTLSo9	+++	+++	+++	++	+	NG	NG
BTLSo10	+++	+++	+++	+++	++	++	+
IBGE3C	+++	++	+++	+++	+++	+++	++

Note: "+++", high growth; "++", moderate growth; "+", low growth, and "NG", no growth. BTN = bacterial isolates from Noakhali; BTCo = bacterial isolates from Cox's Bazar; BTCh = bacterial isolates from Chattogram, and BTL = bacterial isolates from Lakshmipur. IBGE3C was also isolated from Lakshmipur.

Among 41 bacterial isolates, only 3 bacterial isolates provided better growth in 10% and 12% NaCl (*w*/*v*) (Figure 3). Among these 3 isolates, BTCoSo2 was Gram-negative and the other two (BTCoR2 and IBGE3C) were Gram-positive.

2.2. Effect of Bacterial Isolates on Vegetative Growth of BRRIdhan 29 Seedlings

Representative images of variation in root and shoot lengths through the effects of bacteria at 7 days after inoculation are shown in Figures 4 and 5. In root length, both BTCoSo2 and BTCoR2 are significant compared to the control although there was no significant change observed in IBGE3C. All the treatments showed significant changes in

shoot length, root dry weight, and shoot dry weight. Out of these 3 bacteria, the effects of IBGE3C on seedling growth of rice was prominent (Table 2).

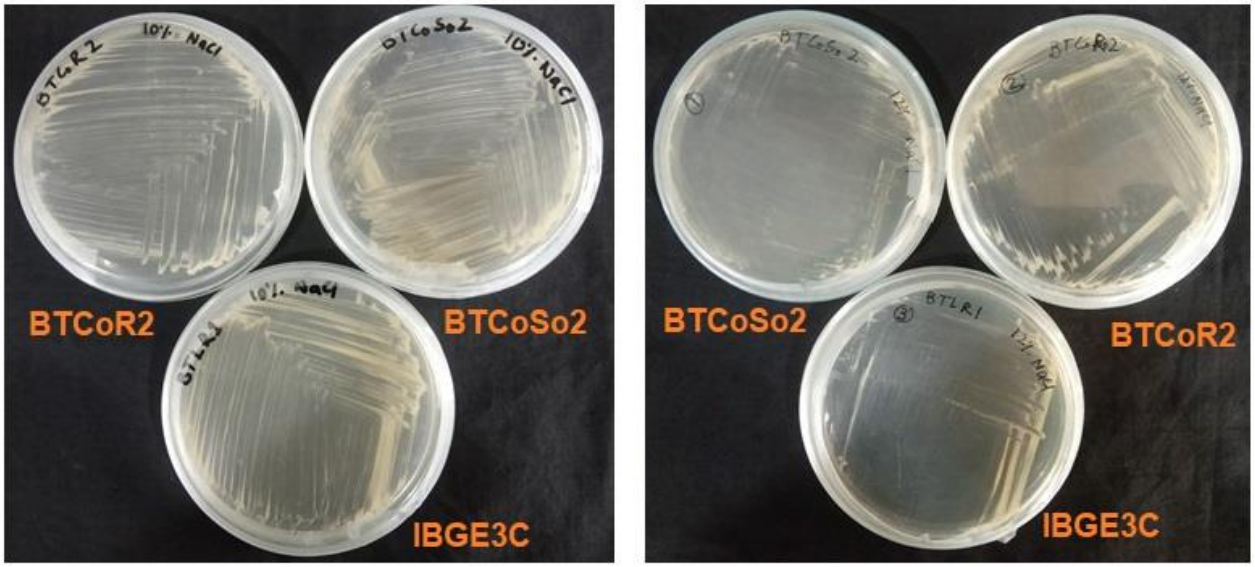

Figure 3. Growth and survival of three potential isolates in nutrient agar medium using higher concentrations of salts including ((**Left**) panel) 10% NaCl (*w*/*v*) and ((**Right**) panel) 12% NaCl (*w*/*v*).

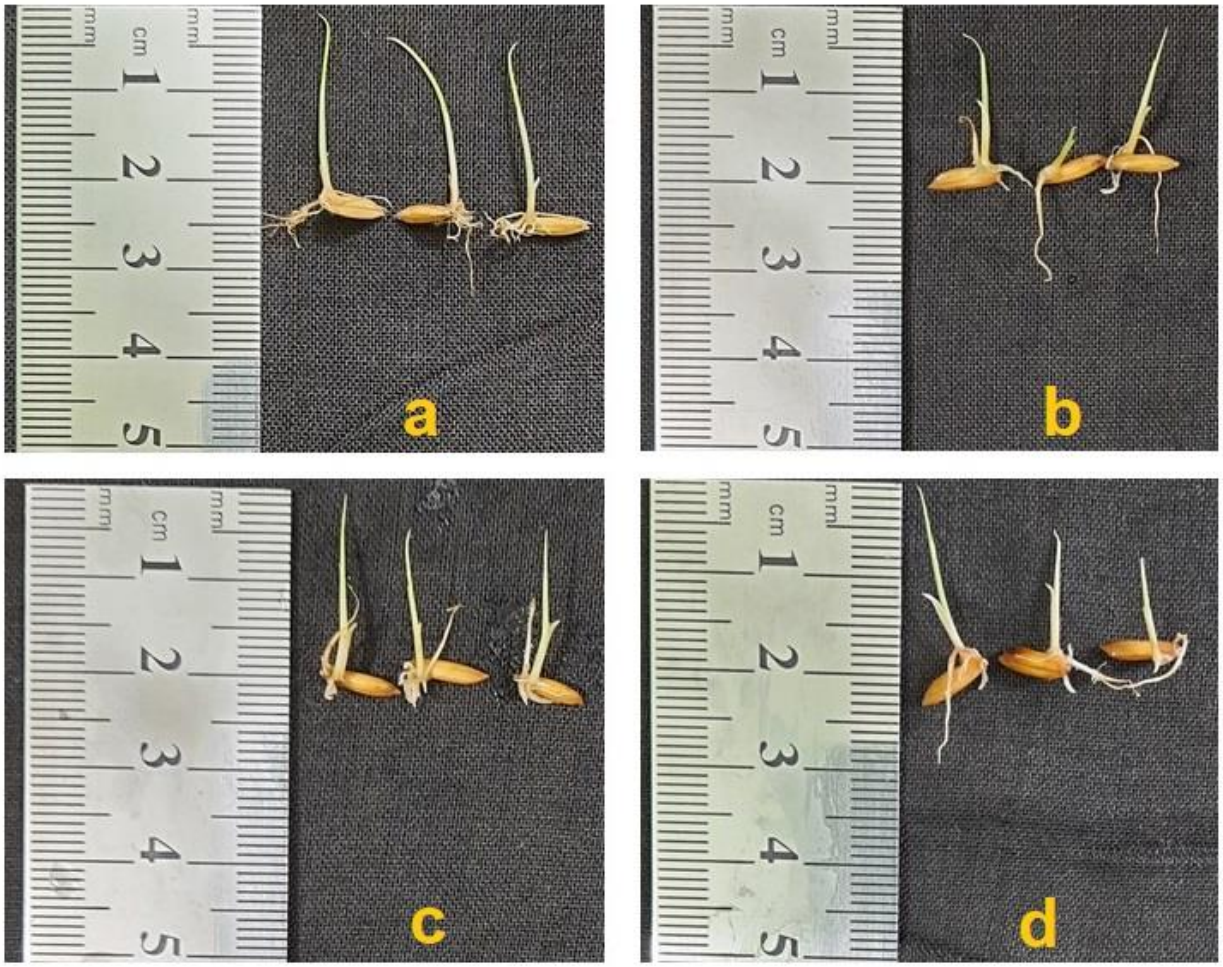

Figure 4. Assessment of BRRI dhan29 rice seedlings treated with three bacterial isolates at 1% salinity. (**a**) Control; (**b**) BTCoSo2; (**c**) BTCoR2, and (**d**) IBGE3C.

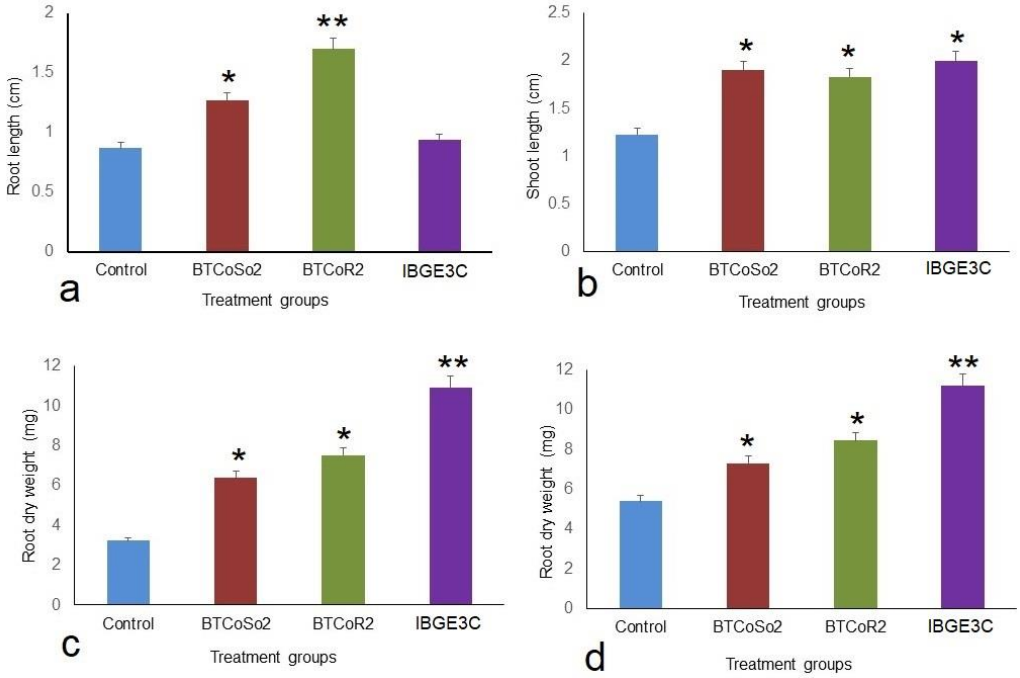

Figure 5. Effects of bacterial isolates on (**a**) root length; (**b**) shoot length; (**c**) root dry weight, and (**d**) shoot dry weight of BRRI dhan29 rice seedlings under 1% salinity. The IBGE3C was identified as *Brevibacterium sediminis*. From left to right, treatments are indicated under columns. Results are expressed in relative length and dry weight compared to control where values are means and bars indicate standard errors (n = 3). * $p < 0.05$ and ** $p < 0.005$ as compared to control.

Table 2. Effects of bacterial isolates on seedling growth of BRRI dhan29 at 1% salinity. Values are mean ± standard error (significance level $\alpha = 0.05$).

Treatment	Seedling Growth *			
	Root Length (cm)	Shoot Length (cm)	Root Dry Weight (mg)	Shoot Dry Weight (mg)
Control	0.87 ± 0.03^{c}	1.23 ± 0.03^{c}	3.23 ± 0.07^{d}	5.4 ± 0.29^{d}
BTCoSo2	1.27 ± 0.09^{b}	1.9 ± 0.12^{ab}	6.4 ± 0.36^{c}	7.33 ± 0.22^{b}
BTCoR2	1.7 ± 0.06^{a}	1.83 ± 0.09^{b}	7.53 ± 0.33^{b}	8.43 ± 0.27^{c}
IBGE3C	0.93 ± 0.09^{c}	2 ± 0.12^{a}	10.93 ± 1.02^{a}	11.2 ± 0.15^{a}

* Any two means having a common letter (in superscript) are not significantly different at the 5% level of significance.

2.3. Effect of Bacterial Isolates on Vegetative Growth of BINAdhan-10 Seedlings

Root and shoot length along with their respective dry weight were recorded after seven days of PGPB inoculation (Figures 6 and 7). In root length, changes were significant in BTCoSo2 and BTCoR2 compared to the control, but no significant changes occurred by the application of *B. sediminis* strain IBGE3C. In shoot length, all three isolates induced significant changes compared to the control, but there were no significant changes among the bacteria treated seedlings. In root and shoot dry weight, significant changes were observed in seedling treated with all the three isolates compared to the control, and there were no significant changes in seedling growth by the isolates of BTCoR2 and BTCoSo2 (Table 3).

2.4. Molecular Identification of the Most Salinity-Tolerant PGPB

A phylogenetic analysis was performed by partial 16S rRNA gene sequencing the most promising salinity-tolerant PGPB isolate, IBGE3C. Following BLAST analysis to search for homology, the sequences together with their closest relatives in GenBank were used to

construct a phylogenetic tree using the maximum-likelihood method (Figure 8). In this study, the isolate IBGE3C showed 99.57% sequence homology with *Brevibacterium sediminis* strain C1 MW564208.1 and *B. sediminis* strain M15.B MZ054436.1 (Figure 8). Therefore, the isolate IBGE3C was tentatively identified as *B. sediminis*.

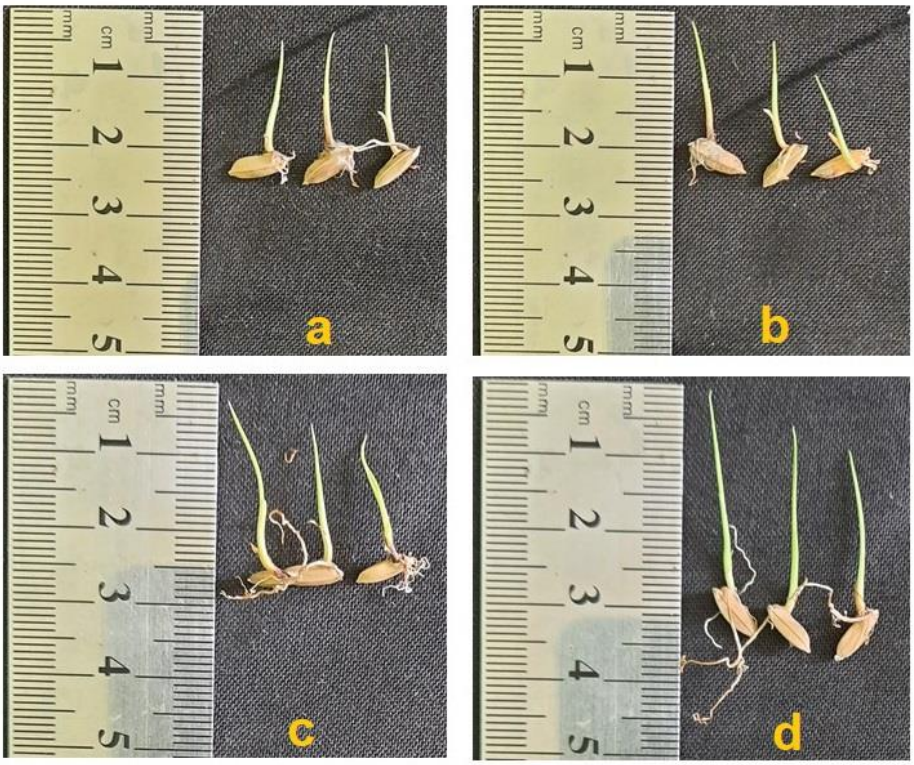

Figure 6. Assessment of BINAdhan-10 rice seedlings treated with three bacterial isolates at 1% salinity. (**a**) Control; (**b**) BTCoSo2; (**c**) BTCoR2, and (**d**) *B. sediminis* strain IBGE3C.

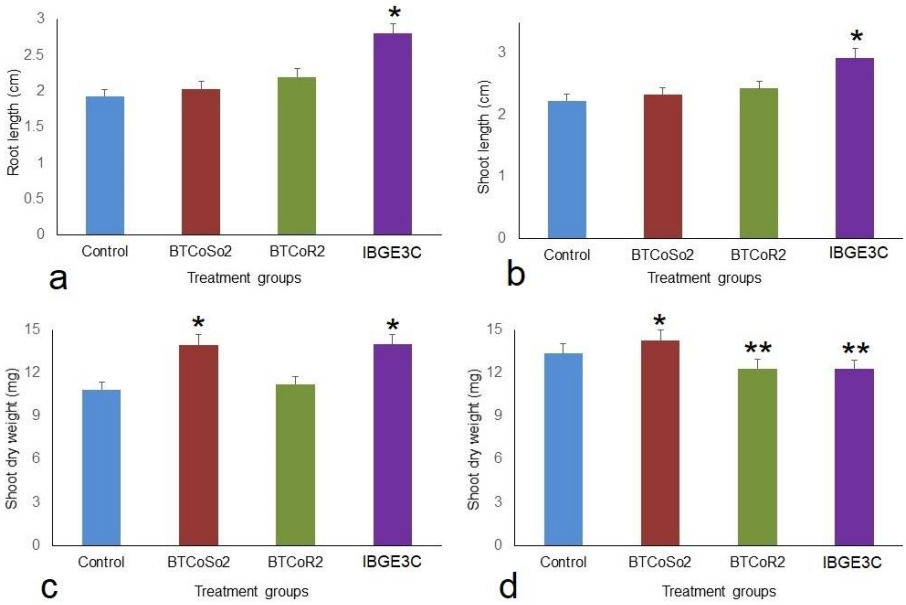

Figure 7. Effects of bacterial isolates on (**a**) root length; (**b**) shoot length; (**c**) root dry weight, and (**d**) shoot dry weight in BINAdhan-10 rice seedlings under 1% salinity. IBGE3C was identified as *Brevibacterium sediminis*. From left to right, treatments are indicated under columns. Results are expressed in relative length and dry weight compared to control where values are means and bars indicate standard errors ($n = 3$). * $p < 0.05$ and ** $p < 0.005$ as compared to control.

Table 3. Effects of bacterial isolates on seedling growth of BINAdhan-10 at 1% salinity. Values are mean and provided with standard error (significance level $\alpha = 0.05$).

Treatment	Seedling Growth *			
	Root Length (cm)	Shoot Length (cm)	Root Dry Weight (mg)	Shoot Dry Weight (mg)
Control	1.93 ± 0.09^{b}	2.23 ± 0.07^{b}	10.83 ± 0.09^{c}	13.33 ± 0.23^{b}
BTCoSo2	2.03 ± 0.12^{ab}	2.33 ± 0.12^{a}	13.93 ± 0.09^{b}	14.23 ± 0.18^{ab}
BTCoR2	2.2 ± 0.15^{a}	2.43 ± 0.07^{a}	11.2 ± 0.15^{b}	12.3 ± 0.21^{ab}
IBGE3C	2.8 ± 0.10^{ab}	2.93 ± 0.09^{a}	13.97 ± 0.12^{a}	12.27 ± 0.20^{a}

* Any two means having a common letter (in superscript) are not significantly different at the 5% level of significance.

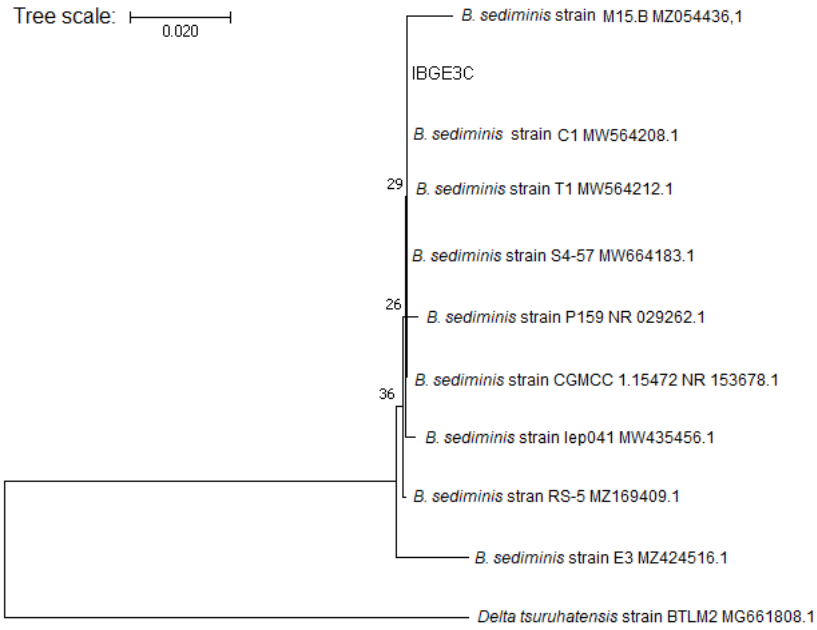

Figure 8. Maximum likelihood (ML) phylogenetic tree of *Brevibacterium sediminis* strain IBGE3C, and some of its closest phylogenetic relatives. Bootstrap values are referred to by the numbers on branches. *Delftia tsuruhatensis* strain BTLM2 MG661808.1 served as an outgroup.

3. Discussion

Salinity is one of the environmental stresses that hamper rice production in the coastal areas of Bangladesh and beyond. To ensure higher crop productivity through environmentally friendly salinity management, the application of the PGPB is a promising biotechnological approach [22,29]. Three bacterial isolates (BTCoSo2, BTCoR2, and *B. sediminis* strain IBGE3C) were selected from the initial forty-one isolates based on their survivability under high salt stress conditions in the culture medium. These isolates displayed higher tolerance to salinity, and were further investigated for their effects on seedling growth of two popular varieties of rice differing from salinity tolerance.

3.1. Isolation and Screening of the Bacterial Isolates

Salinity is a big problem in crop production in the Southern districts of Bangladesh. The situation is aggravating due to the impacts of climate change. Salinity poses a serious threat to the future food security of the country. This study isolated 41 rice-associated bacteria collected from salt-affected areas in Chattogram, Noakhali, Lakshmipur, and Cox's Bazar districts of Bangladesh. Bioassay revealed that these bacteria highly differed in salinity tolerance in vitro. Interestingly, three of them, BTCoSo2, BTCoR2, and *B. sediminis* strain IBGE3C, displayed tolerance of up to 12% of salinity. Isolation of salinity-tolerant bacteria from the salt-adapted plants and soils has previously been reported in several

studies [30–32]. This study for the first time isolated very high salinity-tolerant bacteria associated with rice cultivated in the salinity affected areas of Bangladesh. Enhancement of salinity tolerance in crop plants by the application of the PGPB has been reported earlier [11]. To be effective in enhancing salinity tolerance by the plant probiotic bacteria, the isolate must be salinity tolerant. Therefore, the findings of this experiment of isolation of salinity-tolerant PGPB from rice are encouraging for their evaluation of the growth promotion of rice.

3.2. Effects of Bacterial Isolates on Rice Seedlings in Petri Dish

Three salt stress-tolerant PGPBs isolated from rice remarkably improved the root and shoot growth of rice. Among the two varieties tested, the PGPB isolates showed better performances in seedling growth of salt-tolerant rice variety, BINAdhan-10 (Figures 6 and 7) compared to salt-susceptible rice variety, BRRI dhan29 (Figures 4 and 5). Enhancement of salt tolerance in rice and other crop plants by the application of the PGPB has been reported [33–36]. Growth of *B. sediminis* in nutrient agar medium with up to 20% of NaCl (*w*/*v*) along with the optimum growth at 3.3% NaCl (*w*/*v*) has been reported [37]. In the current study, we observed the salinity tolerance level of *B. sediminis* was up to 12% of NaCl (*w*/*v*) (Figure 3) in a nutrient agar medium. Furthermore, an improvement in growth of rice seedlings by the inoculation of the isolated PGPB from rice cultivated in saline soils was demonstrated by the current study.

In salt-susceptible BRRI dhan29 at 1% salinity, inoculation with highly salt-tolerant *B. sediminis* strain IBGE3C displayed a 7% and 62% increase in the root length and shoot length, respectively, compared to the untreated control. Similarly, root dry weight and shoot dry weight were increased by 238% and 107%, respectively, by the treatment of the same isolate compared to control. Moreover, in salt-tolerant BINAdhan-10 at 1% salinity, *B. sediminis* strain IBGE3C offered 44% and 31% increase in root and shoot length, respectively. Although root dry weight was increased by 28%, shoot dry weight was decreased by 8% in the same condition. In the case of isolate BTCoSo2 on BRRI dhan29 at 1% salinity, it offered a 46% and 54% increase in the root length and shoot length, respectively, and a 97% and 35% increase in the root dry weight and shoot dry weight compared to the untreated control. However, in BINAdhan-10, the same isolate increased the root length and shoot length by 5% and 4%, respectively, compared to control. In addition, root dry weight and shoot weight were increased by 28% and 6%, respectively, by the treatment of that particular isolate. Besides, the isolate BTCoR2 at 1% in BRRI dhan29 increased the root length by 96% and shoot length by 48%; root dry weight and shoot dry weight by 132% and 56%, respectively, compared to the untreated control. Moreover, this isolate on BINA dhan 10 at the same salinity level increased the root length and shoot length by 13% and 8%, respectively; and root dry weight was increased by 3%, and shoot dry weight was decreased by 8%. In this study, the isolated *B. sediminis* strain IBGE3C displayed the best performances in terms of salinity tolerance and seedling growth of rice. However, a further study is needed to test the effects of the rice PGPB on growth and yield of rice in greenhouse and field conditions under varying levels of soil salinity.

3.3. DNA Sequencing and Molecular Identification of the Best Performing Isolate IBGE3C

The 16S rRNA is a gold standard and convenient molecular method for the molecular identification of bacteria. The best performer rice probiotic bacterial isolate, IBGE3C (accession no. MZ573246.1) showed 99.57% sequence similarity with *Brevibacterium sediminis* CGMCC 1.15472 (accession no. NR_153678.1). Although salinity-tolerant *Brevibacterium* spp. have been discovered from some soils [35,37,38] and plant sources [36,38], this study for the first time discovered a high salinity (12% NaCl *w*/*v*)-tolerant *B. sediminis* strain IBGE3C from rice plants cultivated in the salt-affected area (Lakshmipur district) of Bangladesh. The phylogenetic tree based on the 16S rRNA gene sequences showed that the 10 isolates were divided into 3 groups representing a relatively low diversity for the rhizospheric *Brevibacter* species.

The mechanisms of salinity tolerance in bacteria have been reported by Kumar et al. [39]. Although the mechanisms of salinity tolerance and growth promotion of plants by the application of the PGPB have not precisely been elucidated [22], a large body of literature is available on this aspect [35,36,40]. Plant growth-promoting bacteria have both active and passive mechanisms to augment availability of the essential nutrient elements for plants and improve soil health [41]. PGPB minimizes the detrimental effects of salinity by retaining the required ratio of Na^+/K^+ through excreting exopolysaccharides (EPS) that consequently secure their survival under salt-stressed conditions [42,43]. Similar to other PGPB, *Brevibacterium* spp. can produce significant amounts of ACC deaminase [36], and it has been found that inoculation of plants with ACC deaminase-producing bacteria increases root biomass and produce longer roots [11,44]. High production of antioxidant enzymes reduces the generation of hydrogen peroxide under salt stress [45]. PGPB directly help a plant's salt tolerance and hence, improve growth by producing IAA [46,47]. Moreover, the sodium ion (Na^+) binding capacity of PGPB maintains cellular turgidity and defends chloroplast from adverse impacts of salinity and thus increasing photosynthesis, chlorophyll synthesis, and plant growth under salt-stressed conditions [48,49]. However, further in silico and laboratory analyses of the whole-genome sequence of this salinity-tolerant bacteria would result in interesting insights into its plant growth promoting and salinity tolerance mechanism that are needed for their practical application in rice. This study for the first time demonstrated that a very high salt-tolerant PGPB isolate, *B. sediminis* IBGE3C, native to rice plants cultivated in salt-affected areas, enhanced seedling growth of rice under varying levels of salinity.

4. Materials and Methods

4.1. Plant Samples Collection

Two rice varieties were selected; one was salinity susceptible (BRRI dhan29) and the other was salinity tolerant (BINAdhan-10). Rice plant samples were collected from four coastal districts of Bangladesh (Chattogram, Cox's Bazar, Lakshmipur, and Noakhali). Sample collection areas were selected based on salinity in the soil. The variety of plant samples collected from Chattogram and Noakhali were cv. BRRI dhan29. In addition, Cox's Bazar and Lakshmipur plant samples were cv. BRRI dhan28. All plant samples were salt-susceptible and the plant varieties were confirmed by corresponding field farmers. Seeds were collected from the Bangladesh Rice Research Institute (BRRI), regional station, Cumilla, and Bangladesh Institute of Nuclear Agriculture (BINA), substation in Cumilla. The seed samples were selected based on salinity tolerance. Five rice plant samples were randomly collected from the corresponding field of each of the four districts mentioned earlier. The rice seedlings were uprooted having the rhizospheric soils and kept in a sterile ziplock polybag with marking to avoid any cross-contamination during sampling [49].

4.2. Isolation and Screening of Bacteria

For the collection of potential salt-tolerant bacteria, different plant parts such as the leaf, root, and rhizospheric soil were taken. The bacteria were isolated from plant parts using protocols described earlier [11,50]. Six-fold serial dilution was prepared in autoclaved water [51]. Of them, fifty microliter aliquots from a particular dilution were taken through a pipette and spread on nutrient broth agar (NBA) plates and then placed in the incubator for 24 h at 26 °C ± 1 °C. After incubation, different bacterial colonies were isolated and purified on an NBA medium. For salt tolerance evaluation, the isolated bacteria were grown in NBA medium with various doses of sodium chloride (NaCl) (2%, 4%, 6%, 8%, 10%, and 12% *w*/*v*). Then, the growth of the bacteria in the medium with different concentrations of salt was observed and recorded.

For the identification of Gram-positive and Gram-negative bacteria, a loopful of bacteria was taken onto a glass slide and mixed with a drop of 3% KOH solution to make a smear [52]. After a few minutes, their characteristics were observed. A positive reaction is represented by the formation of thread-like mass, whereas KOH positives are Gram-

positive and the KOH negatives are Gram-negative [53]. The current study involved three replications of each experiment.

4.3. Preparation of Bacterial Inocula

Bacterial isolates were cultured in 250 mL conical flasks containing 200 mL NB broth medium on a shaker at 120 rpm for 72 h at 27 °C. To collect bacterial cells, the broth was centrifuged at 14,000 rpm for 1 min at 4 °C, and two times washed with SDW (Sterile Distilled Water). The bacterial pellets were suspended in around 1 mL SDW and vortex for 45 s before using for the seed treatment.

4.4. Seed Treatment with Bacteria

One gram of surface-sterilized seeds was soaked into bacterial suspension. The bacteria treated seeds were dried overnight at room temperature to ensure a better coating of the seeds with bacteria [54].

4.5. Seedling Assay

The inoculated seeds were placed on a Petri dish containing a water-soaked sterilized filter paper [54–57]. After the germination of seeds, the seedlings were allowed to grow for one week. The seedlings were watered on alternate days. The seedling assay was carried out under varying levels of salinity such as 0%, 0.2%, 0.4%, 0.6%, 0.8%, 1%, 2%, 4%, 6%, 8%, and 10% NaCl (*w*/*v*). However, data were taken from the 0% to 1% level of salinity because the germination of rice seeds sharply declined from 1% salinity and no germination occurred above 2% salt concentration and so on.

4.6. Genomic DNA Extraction, Ribosomal Gene (16S rRNA) Sequencing, and Data Analysis

Wizard®'s Genomic DNA Purification Kit (Cat. No A1120) was used for the extraction of DNA from pure culture of bacteria. DNA quantity and purity were determined with NanoDrop 2000 (ThermoFisher, Waltham, MA, USA) by measuring 260/280 absorbance ratios, and stored at −20 °C. Following DNA extraction, PCR amplification was carried out targeting the ribosomal (16S rRNA) gene fragments. The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-CTACGGCTACCTTGTTACGA-3′) [55–58]. The individual PCR reaction mixture contained nuclease-free water, buffer, dNTPs, forward primer, reverse primer, Taq polymerase, and sample DNA. PCR amplification was performed in the Veriti® 96-Well thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR product was sequenced using Sanger dideoxy sequencing method. Using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 for the larger datasets [59], the nucleotide sequence of the corresponding isolates was visualized. In order to search for nucleotide sequences similarity, Genbank databases were used by online program nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (accessed on 21 November 2021)). Closely related sequences were retrieved from NCBI and subjected to multiple sequence alignment by ClustalW program. Trimmomatic program (version 0.39) was used to estimate the quality of each sequence, edit, and trim poor quality sequences [60]. A maximum-likelihood tree was generated by MEGA 7.0 software using default parameters, and visualized by iTOL v5.6.1 [61]. Nodal confidence in the resulting phylogenetic relationships was assessed using the bootstrap test (1000 replicates). The sequences of the strain IBGE3C were submitted in NCBI [https://www.ncbi.nlm.nih.gov (accessed on 21 November 2021)], under the GenBank accession numbers: MZ573246.1.

4.7. Statistical Analysis

The statistical methods include one-way analysis of variance (ANOVA) using Minitab (version 2017) Fisher's Least Significant Difference (LSD) method for grouping analysis, and MS excel (MS version 2017) for preparing graphs and other calculations. A two-tailed Student's *t*-test was used for the comparisons across the means of four groups, and p values < 0.05 were considered as statistically significant.

5. Conclusions

The mutualistic association between PGPB and plants is well established. This study scrutinized three highly salinity-tolerant bacterial isolates obtained from rice cultivated in the saline soils of Bangladesh. These bacterial isolates, viz., BTCoSo2, BTCoR2, and IBGE3C were evaluated on growth promotion of rice seedlings. In general, these elite bacterial strains improved the seedling growth of rice varieties (BRRI dhan29 and BINAdhan-10) with varying levels of salinity tolerance. However, the effects of the bacteria were more pronounced in BINAdhan-10. Among the bacterial isolates, the best performing one, strain IBGE3C, was tentatively identified as *B. sediminis* using 16S rRNA gene sequencing. The identification of an isolate of salinity-tolerant and rice growth-promoting *B. sediminis* strain IBGE3C is the first evidence in Bangladesh. The *B. sediminis* isolated in this study showed PGP abilities, which is potential to be used as PGPB. This is a pre-field study under pilot phase investigation of some potential salt-tolerant PGPB from rice. The results indicate that our three salinity tolerant isolates could be useful in the formulation of new PGPB inoculants. Although the findings of this study are limited to in vitro bioassay, these findings could serve as the bench mark clues for further investigation of the performances and mode of action of these bacterial isolates in promoting growth and increasing the yield of rice plants under adverse field conditions in the salinity-prone coastal regions of Bangladesh. Further studies are also needed for field evaluation of the performance of the particular strain of *B. sediminis* on rice in the saline soils of Bangladesh.

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Abbreviations

EC	Electrical conductivity
ECe	Electrical conductivity of the saturation extract
RPM	Rotation per minute
NaCl	Sodium chloride
KOH	Potassium hydroxide
SDW	Sterile distilled water
ACC	1-aminocyclopropane-1-carboxylic acid
IAA	Indole-3-acetic acid
NBA	Nutrient broth agar
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
BINA	Bangladesh Institute of Nuclear Agriculture
BRRI	Bangladesh Rice Research Institute
IBGE	Institute of Biotechnology and Genetic Engineering
BSMRAU	Bangabandhu Sheikh Mujibur Rahman Agricultural University

NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
ANOVA	Analysis of variance
LSD	Least Significant Difference

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