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Characterization and crop production efficiency of diazotrophic bacterial isolates from coastal saline soils

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ABSTRACT

Use of eco-friendly area specific salt tolerant bioinoculants is better alternatives to chemical fertilizer for sustainable agriculture in coastal saline soils. We isolated diverse groups of diazotrophic bacteria from coastal saline soils of different forest and agricultural lands in the Sundarbans, West Bengal, India, to study their effect on crop productivity in saline soils. Phenotypic, biochemical and molecular identifications of the isolates were performed. The isolates produced indole acetic acid, phosphatase, and solubilized insoluble phosphates. Sequence analysis of 16S rDNA identified the SUND_BDU1 strain as *Agrobacterium* and the strains SUND_LM2, Can4 and Can6 belonging to the genus *Bacillus.* The ARA activity, dinitrogen fixation and presence of *nifH* genes indicated they were diazotrophs. Field trials with these strains as bioinoculants were carried out during 2007–2009, with rice during August–December followed by Lady's finger during April–June. Microplots, amended with FYM inoculated with four bioinoculants individually were compared against sole FYM (5 t ha^{-1}) and a sole chemical fertilizer (60:30:30 kg ha^{-1} NPK) treated plot. The strain Can6 was by far the best performer in respect of yield attributes and productivity of studied crops.

1. Introduction

In the context of increasing international concern for food and environmental quality, the use of plant growth-promoting rhizobacteria (PGPR) for reducing chemical inputs in agriculture is a potentially important issue. The PGPR were applied to various crops to enhance growth, seed emergence and crop yield, and a few such applications have been commercialized (Dey et al. 2004; Herman et al. 2008; Minorsky 2008). Diazotrophic bacteria are also PGPR, because of their competitive advantage in C-rich and N-poor environments (Kennedy et al. 2004). Diazotrophic bacteria were reported to secrete growth promoting hormones like auxin, gibberellin and cytokinin into their culture media (Fuentes-Ramirez et al. 1993). By virtue of such attributes, pretreatment of seeds with a suspension of *Azotobacter* was shown to improve seed germination and plant growth (Ravikumar et al. 2004). Interestingly, diazotrophic microorganisms showing phosphate solubilizing activity are rarely reported (Verma et al. 2001; Loganathan and Nair 2004). Several reports revealed that inoculation with free living diazotrophs like *Azotobacter*, *Pseudomonas* and *Azospirillum* increased the yield of rice by 20–55% (Yanni and El-Fattah 1999; Mirza et al. 2006; Balandreau 2002) and a strain of diazotrophic *Burkholderia* increased the rice plant biomass by 69% (Kennedy et al. 2004).

Salinity is one of the most serious environmental problems influencing crop growth throughout the world (Ravikumar 2008). In India, out of an estimated area of 187.7 million ha of total degraded lands, 8.1 million ha are salt affected in which 3.1 million ha are in the coastal regions (Tripathi et al. 2007). Among the states of India, West Bengal has the largest area (0.82 million ha) of salt affected soil in coastal region. N input for crop production is very important particularly in saline habitats which are N-poor. An increasing supply of N through dinitrogen fixation may increase crop production in saline habitats (Zahran 1997). Salt tolerant symbiotic nitrogen fixers like *Rhizobium* increases crop productivity in saline soils (Zahran 1999) but the potential of free living diazotrophs is not well understood (Chavada et al. 2010; Ravikumar et al. 2004).

Considering the efficacy, input cost and environmental safety, use of chemical fertilizers for crop production in coastal saline soil is not a sound proposition. We hypothesized that the identification and application of the most efficient area specific microbial inoculants may help in accruing benefit to agricultural crop production in the problematic saline soil. However, there is a controversy in the literature as to the competitive ability of the introduced strains to establish against native populations (Wani 1992).

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We report here the identification of four diazotrophs isolated from the representative forest and agricultural ecosystems of the naturally occurring saline habitats of the Sundarbans. The study included in-depth assay of the phenotypic, biochemical and molecular characteristics and crop production efficiency of these isolates when used as bioinoculants.

2. Materials and methods

2.1. Isolation and purification of free-living diazotrophic bacteria

Nitrogen-free mineral salt-yeast extract broth (Barua et al. 2008) was used for enrichment followed by purification on the same solid media. The media contained yeast extract (100 mg l^{-1}), to enhance N_2 fixation (Reis et al. 2004). NaCl concentration was maintained at 1% (w/v). 40 pure cultures were obtained and tested for their N fixing ability (data not shown). Four pure cultures with higher N_2 fixing ability were selected for future use in field trial. The SUND_BDU1 and SUND_LM2 strains were isolated from forest soils of the Sunderbans (21°32′–22°40′N and 88°85′–89°0′E), while Can4 and Can6 strains were isolated from agricultural fields of Central Soil Salinity Research Institute (CSSRI), Canning Town (20°15′N and 80°40′E), West Bengal, India. The cultures were maintained in same agar slants with 1% NaCl.

2.2. Phenotypic and biochemical characterization of the isolates

Phenotypic characters of the isolates like cellular morphology, Gram reaction and capsule and spore formation were carried out. Biochemical characteristics of the isolates like sugar utilization pattern, starch hydrolyzing activity, catalase activity, NO_3^- utilization, IMViC analysis, gelatinase activity and ability to grow on TSI agar slant were determined by standard procedures (Holtz 1993).

2.3. Acetylene reduction assay (ARA)

The isolates and *Azotobacter vinelandii* (MTCC 2460) used as the standard organism were incubated in 3 ml nitrogen-free mineral salt-yeast extract broth containing 1% NaCl for 72 h at 29 °C in 7 ml Becton Dickinson Vacutainer tubes stoppered with cotton plugs. After visible growth, cotton plugs were aseptically exchanged with rubber stoppers and the headspace air was replaced with 10% (by volume) of high purity C_2H_2 gas by hypodermic syringe. The C_2H_4 production was measured after 72 h incubation of the tubes in dark at 29 °C (Rao et al. 1983). Tubes without C_2H_2 served as controls. For the determination of C_2H_4, 0.5 ml of the gas phase from each tube was injected into gas chromatograph fitted with flame ionization detector (FID).

2.4. Determination of nitrogen fixing efficiency

The isolates and *A. vinelandii* (MTCC 2460) were grown in 50 ml nitrogen free mineral salt-yeast extract-broth containing 1% NaCl, for 7 days at 30 °C, followed by the determination of nitrogen in the cultures as well as the uninoculated blanks, by macro Kjeldahl method (Allen 1957).

2.5. Determination of phosphate solubilization and phosphatase activity

Cells were grown in Pikovskaya broth containing tricalcium phosphate with 1% NaCl, at 30 °C for 72 h. Solubilization of aluminum phosphate and β-glycerophosphate was determined by replacing tricalcium phosphate with other phosphates in the same broth. Phosphorus in the culture filtrate was determined by colorimetric ascorbic acid method (John 1970).

Acid phosphatase activity of the isolates was determined in cell free culture broth amended with insoluble inorganic and organic phosphorus sources (Tabatabai and Bremner 1969). The experiment was carried out in triplicate by incubating the cultures for 72 h in media containing 1% NaCl.

2.6. Estimation of indole-3-acetic acid (IAA) production

Cells were grown in nitrogen free mineral salt-yeast extract-broth containing 1% NaCl supplemented with L-tryptophan (100 mg l^{-1}) at 30 °C for 72 h. Cell free extracts were assayed in triplicates for the production of auxins (IAA equivalents) according to Gordon and Weber (1951).

2.7. Extraction of genomic DNA

Cells from 5 ml of overnight culture were centrifuged at 10,000 × *g* for 15 min and resuspended in 2 ml extraction buffer (10 mM Tris·HCl, 5 mM EDTA). After treatment with lysozyme (1 mg ml^{-1}) followed by sodium dodecyl sulfate (1% for 60 min at 37 °C), 0.3 ml of cetyltrimethylammonium bromide was added. The DNA was extracted by phenol and chloroform, precipitated with isopropanol, and dissolved in Tris–EDTA buffer (20 mM Tris and 5 mM EDTA) (Ghosh et al. 2007). The DNA preparation was treated with RNase A, and the DNA concentration was estimated by visual examination of ethidium bromide-stained agarose gel as well as by spectrophotometric examination. DNA was stored at −20 °C for further use.

2.8. Amplification of DNA segments

A 977 bp portion of the 16S rDNA gene from selected strains was amplified by the polymerase chain reaction using bacterial universal primers 515F (5′-GTGCCAGCAGCCGCGGTAA-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The *nifH* gene amplification was done using degenerate *nifH* specific forward primer, PolF (5′-**TGCGAYCCSAARGCBGACTC**-3′) and reverse primer, PolR (5′-**ATSGCCATCATYTCRCCGGA**-3′) based on *A. vinelandii nifH* coding sequence [M20568] to generate a 360 bp region (Poly et al. 2001a,b). The PCR mix consisted of dNTPs at 200 mM each, 0.25 μM of each primer, 2.5 mM $MgCl_2$, 1× PCR buffer and 2 U of Taq DNA polymerase (Promega, USA). For 16S rDNA or *nifH* amplification, approximately 20–50 ng or 100 ng of genomic DNA from each of the isolates was used per 50 μl PCR, respectively. The following cycling conditions were used for 16S rDNA: 94 °C for 3 min, followed by 29 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 7 min (Ghosh et al. 2007). Amplification protocol for *nifH* was as follows: 94 °C for 3 min, followed by 29 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The PCR products were run in 0.8% agarose gel and stained with ethidium bromide. The DNA bands were purified from agarose gels with the Gel Extraction Kit (Promega, USA) as per manufacturer's protocol.

2.9. Sequencing and phylogenetic analysis of the 16S rDNA and nifH fragments

The PCR amplified regions from 16S rDNA and *nifH* genes were sequenced in ABI 3100 Genetic Analyzer with primers 515F and PolF, respectively. Sequencing reaction was performed using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The partial 16S rDNA sequences of the isolated strains were compared with those available in the public databases. Nucleotide sequences were compared with sequences in GenBank database by BLAST-N algorithm (Altschul et al. 1997) to identify sequences with a high degree

Table 1
Dinitrogen fixing potential and fixation and plant growth promoting activities of the bacterial strains at 1% NaCl in culture media.

Strain code	ARA[a] (nmol of C_2H_4 formed ml^{-1} culture media 72 h^{-1})	Dinitrogen fixation (mg N_2 fixed 50 ml^{-1} culture media)	Indole acetic acid production (μg ml^{-1} culture filtrate)	Tricalcium phosphate		Aluminum phosphate		β-Glycerophosphate	
				Phosphate solubilization (%)	Acid phosphatase activity (μmol pnp ml^{-1} culture filtrate h^{-1})	Phosphate solubilization (%)	Acid phosphatase activity (μmol pnp ml^{-1} culture filtrate h^{-1})	Phosphate solubilization (%)	Acid phosphatase activity (μmol pnp ml^{-1} culture filtrate h^{-1})
SUND_BDU1	47.21 b	4.08 a	11.92 c	60.09 b	0.26 b	2.54 b	0.12 b	9.21 c	0.143 a
SUND_LM2	4.55 c	4.08 a	2.79 d	53.87 c	0.14 d	1.25 b	0.04 c	12.32 b	0.076 b
Can4	1.72 d	4.08 a	2.07 d	74.77 a	0.48 a	6.83 a	0.21 a	12.87 b	0.161 a
Can6	55.60 a	4.76 a	21.85 b	52.41 c	0.20 c	2.23 b	0.12 b	20.40 a	0.179 a
Azotobacter vinelandii (MTCC2460)	5.03 c	1.64 b	23.16 a	–	–	–	–	–	–

Figures denoted by same alphabets are statistically similar at 5% probability level by DMRT.
[a] Acetylene reduction assay; culture media contained 1% NaCl.

of similarity. Phylogenetic trees of both 16S rDNA and *nifH* gene sequences were generated using the neighbor-joining algorithms using the p-distance model (Saitou and Nei 1987) in Mega IV software (Tamura et al. 2007). The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 500 bootstrap replicates. For 16S rDNA based phylogenetic tree, 52 reference sequences from GenBank were used. The optimal tree with the sum of branch length of 0.70065918 is shown. For *nifH* gene based phylogenetic tree analysis, 36 reference sequences from GenBank were compared with *Anabena* sp. 9109 (AY768419) as outgroup. The optimal tree with the sum of branch length of 4.38601444 is shown. The percentage of replicate for both the trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 319 positions for 16S rDNA and 143 positions for *nifH* based tree in the final dataset. Concordance of such analysis was also verified with maximum parsimony algorithm.

2.10. Field trial

The field experiments were carried out at the experimental farm of CSSRI, Canning Town, in the years 2007–2008 and 2008–2009 with rice (*Oryza sativa* L.) followed by Lady's finger (*Abelmoschus esculentus* L.). Rice (cv "Swarna") was transplanted in the month of August and harvested in the month of December. Lady's finger (cv "F1 Hybrid 016") was sown in the month of April and harvested in the month of June. The experiment was carried out in fixed plots of size 4 m^2 laid out in a randomized block design with seven treatments each replicated thrice. The physico-chemical properties of the experimental soil were: pH 6.2; EC_e 3.6 dS m^{-1}; organic carbon 0.72%; total N 0.06%; available P 12.8 kg ha^{-1} and available K 452 kg ha^{-1}. The treatments consisted of T1, control; T2, application of farm yard manure (FYM)@5 t ha^{-1}; T3, application of NPK@60:30:30 kg ha^{-1} in the form of urea, superphosphate and muriate of potash, respectively; T4, application of SUND_BDU1 along with FYM@5 t ha^{-1}; T5, application of SUND_LM2 along with FYM@5 t ha^{-1}; T6, application of Can4 along with FYM@5 t ha^{-1}; T7, application of Can6 along with FYM@5 t ha^{-1}. The FYM used contained 29.2% organic carbon, 0.58% total N, 0.19% P and 0.8% K.

The bioinoculants were prepared by mass culturing the selected strains in nitrogen-free mineral salt yeast extract broth supplemented with 1% NaCl for 72 h. A volume of 500 ml of this culture containing 1×10^9 cfu ml^{-1} of each isolate was used. The root portion of 21 day old rice seedlings was dipped in the culture media for 20 min and transplanted immediately. For the control, the sole FYM amended and chemical fertilizer treated plots, the seedlings were dipped in uninoculated culture broth. In case of Lady's finger, the bioinoculants were applied by drenching the pits after 21 days of emergence of the seedlings with a knapsack sprayer in the root zone according to the treatments. The control, solely FYM and chemical fertilizer treated plots were not inoculated.

Rice seedlings were transplanted in rows 20 cm apart and 15 cm distance was maintained between hills in a row for a plot size of 4 m^2, so that each plot contained 90 hills. The chemical fertilizers were applied in the respective plots at the time of transplanting, while the requisite quantities of FYM were applied 7 days prior to transplantation of rice according to the treatments. The Lady's finger seeds were sown in pits 50 cm apart from each other totaling 30 pits per plot. The fertilizers and FYM were applied treatment-wise in same quantities applied to rice.

In case of rice, three hills were randomly collected at harvest from each replicated plot and the following crop measurements

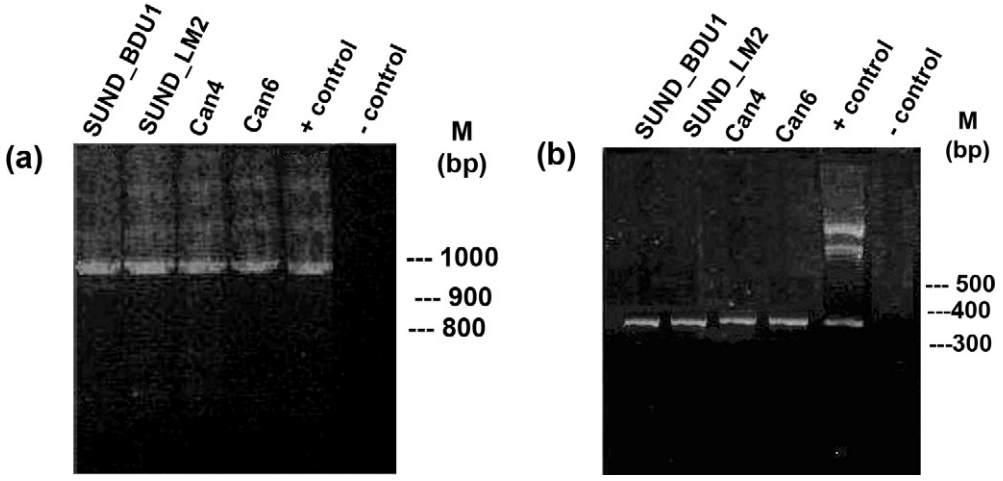

Fig. 1. Agarose gel electrophoresis of PCR products obtained by amplifying (a) 16S rDNA and (b) *nifH* from the genomic DNA of bacterial isolates. Amplified products from all the four isolates (SUND_BDU1, SUND_LM2, Can4 and Can6) were compared with the same amplicon from *A. vinelandii* used as positive control.

were performed: plant height, number of effective tillers per hill, panicle length, thousand grains weight, straw yield and grain yield.

In case of Lady's finger, biometric observations that of plant height, number of leaves per plant and fresh fruit yield were recorded. The percent nitrogen content of both rice and Lady's finger were determined by the method suggested by Kalra and Maynard (1991).

2.11. Statistical analyses

Analysis of variance (ANOVA) between the biochemical parameters of the inoculants was carried out by completely randomized design (CRD) while the same between the crop biometric experimental field measurements was carried out by randomized block design (RBD). The least significant (LSD) difference test was applied to evaluate the significance of difference between individual treatments at 5% probability level using Duncan Multiple Range Test with SPSS statistical software (version 11).

3. Results

Altogether 40 pure cultures of diazotrophic organisms were isolated from coastal saline soils and tested for their nitrogen fixation and nitrogen fixing potential, IAA production and phosphate solubilization potential. Based on such data, four potent isolates were selected for further study.

3.1. Morphology and biochemical characteristics

The strain SUND_BDU1 was rod shaped, Gram negative and non spore former. The other three strains SUND_LM2, Can4 and Can6 were rod shaped, Gram positive and capable of forming spore. All the strains produced capsules. The strains could use glucose, sucrose, starch, mannitol, nitrate and citrate, but they were indole, methyl red and VP negative. The organisms showed a positive test for catalase and gelatinase. Growth of the organisms on TSI agar slants showed alkaline reaction (data not shown).

3.2. The ARA, nitrogen fixation, IAA production, phosphate solubilization and phosphatase activity

Table 1 summarizes the properties of the isolates when grown under conditions that could be important for plant growth in saline coastal soil. The organisms were screened by growing them in culture media containing 1% NaCl, so that the organisms could be used as potential microbial inoculants for coastal agriculture. The ARA showed significant variation between the isolates. The strain Can6 recorded the highest value and others could be ranked as SUND_BDU1 > SUND_LM2 > Can4 with the strain SUND_LM2 being statistically similar to the standard strain of *A. vinelandii* (MTCC 2460). All the isolates showed higher nitrogen fixation compared to the standard strain with Can6 again recording the highest value.

The IAA production (2.07–21.85 mg ml^{-1} culture filtrates) by the isolates was lower compared to the standard strain *A. vinelandii* (MTCC 2460). Can6 recorded the highest value followed by SUND_BDU1. The isolates could utilize all the phosphate sources under study, with the highest percentage from tricalcium phosphate, followed by β-glycerophosphate and aluminum phosphate. The Can4 strain showed the highest activity on both calcium and aluminum phosphates. However, Can6 recorded the highest activity with β-glycerophosphate, followed by Can4 > SUND_LM2 > SUND_BDU1. The pH of the culture filtrate (6.4–6.7) in the presence of all the three phosphate sources was in the acidic range after incubation (data not shown). As acid phosphatase predominates in acidic media (Juma and Tabatabai 1977), only this activity of the culture filtrate was measured. The acid phosphatase activity in the culture filtrate of the strain Can4 was higher than the other strains in both tricalcium phosphate and aluminum phosphate substrates. With β-glycerophosphate, the phosphatase activities of Can4, Can6 and SUND_BDU1 were similar and significantly higher than that of SUND_LM2. The standard strain of *A. vinelandii* (MTCC 2460) failed to show any kind of phosphatase activity or phosphate solubilization.

3.3. Analysis of 16S rDNA and nifH fragments

Amplification of the 16S rDNA and *nifH* gene segments yielded the expected 977 bp and 360 bp products (Fig. 1). In both the cases DNA isolated from *A. vinelandii* was used as positive control.

Based on their 16S rDNA gene sequences, isolates were classified as alphaproteobacteria (SUND_BDU1) or firmicutes (SUND_LM2, Can4, Can6). More specifically, SUND_BDU1 showed 99% sequence similarity with members of the genus *Agrobacterium*. Isolates SUND_LM2, Can4 and Can6 were assigned to the genus *Bacillus*. Concordantly comparative sequence analysis of the 16S rDNA gene fragment showed that SUND_LM2, Can4 and Can6, along with the genus *Bacillus*, form a monophyletic cluster (Fig. 2). The strain SUND_BDU1 however clustered with members of the genus *Agrobacterium*. Trees constructed with different methods, including neighbor joining and maximum parsimony provided support

Table 2
Molecular characterization of the isolates.

Code of the strain	Length of the 16S rDNA sequence (bp)	GenBank accession number	Most closely related organism based on 16S rDNA sequence analysis	Accession number of the closely related organism	% similarity	nifH accession number
SUND_BDU1	551	EU732656	*Agrobacterium* sp.	EU652867	99	GQ891110
SUND_LM2	595	EU831304	*Bacillus* sp.	DQ523735	99	GQ411215
Can4	565	EU831294	*Bacillus* sp.	DQ523735	100	GQ411207
Can6	596	EU831296	*Bacillus* sp.	EF667987	99	GQ891112

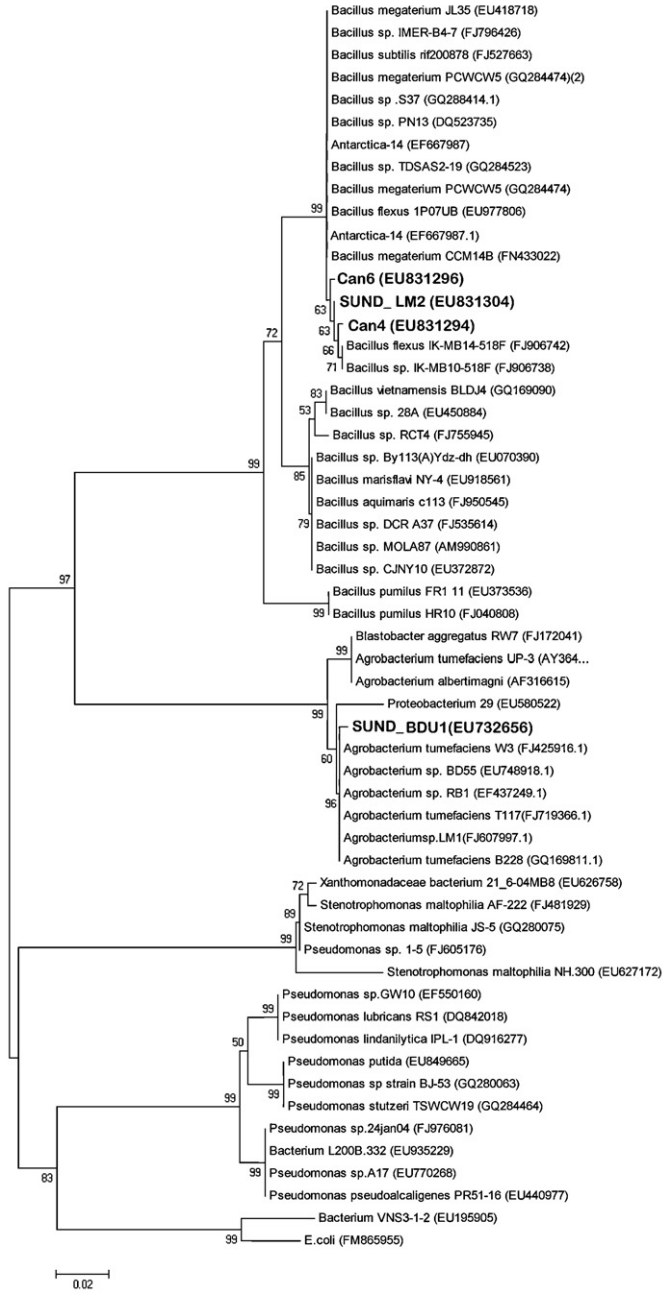

Fig. 2. Neighbor joining phylogenetic tree based on partial 16S rRNA gene sequences. Saline soil isolates (SUND_BDU1, SUND_LM2, Can4 and Can6) were analyzed with other bacterial species from the database with *E. coli* used as outgroup. The database accession numbers are indicated after the bacterial names. The scale bar represents 0.02 substitutions per site. The bootstrap values are presented above the nodes (values below 50% are not shown).

for the coherence of these clusters. The preliminary characterization of these isolates also supports this placement based on of their Gram reaction and other morphologic and biochemical characters. The GenBank accession numbers for these sequences are provided in Table 2.

Previous studies have reported possible role of horizontal gene transfer in nitrogenase evolution obtained by comparing nifH phylogenies to ribosomal RNA phylogenies (Chien and Zinder 1996; Zehr et al. 2003). Phylogenetic analysis of *nifH* in SUND_BDU1 by neighbor joining and maximum parsimony algorithms demonstrates its clustering with *Paenibacillus*, a Gram-positive, facultative anaerobic diazotroph and *Agrobacterium tumefaciens*. However the *nifH* sequences present in SUND_LM2, Can6 and Can4 were phylogenetically heterogeneous (Fig. 3). While the SUND_LM2 *nifH* sequence is clustered with known *nifH* sequences from *Bacillus*, the Can4 and Can6 sequences were largely unclustered but derived from a common ancestor.

3.4. Field trial

Two years pooled data obtained from the field trials in respect of various growth and yield parameters of rice during two successive monsoon seasons is presented in Table 3. Differences between the inoculated treatments and exclusive fertilizer or farmyard manure application were evident. The exclusive fertilizer (T3) treatment was the most effective between the treatments in significantly increasing the measured parameters of rice. The tallest plant could be observed with the T3 treatment (102.67 cm) and the shortest in the control treatment (80 cm). The plant heights were statistically similar in both the inoculated and exclusive FYM (T2) treatments. The T3 treatment produced the most number of effective tillers hill^{-1} (18) and maximum panicle length (27 cm), which was statistically and significantly higher than the other treatments. The T2 treatment and the inoculated treatments (T4, T5, T6, and T7) produced statistically similar numbers of effective tillers hill^{-1}. It was observed that the number of effective tillers, on an average, increased by 12 and 60% in the inoculated treatments than the exclusive FYM (T2) and control treatments, respectively. The thousand grains weight was the highest in the T3 treatment (28.85 g). Among the inoculated treatments, although the thousand grains weight of the Can6 inoculated treatment (T7) was higher, it was statistically similar to the T5 and T6 treatments. There were large variations in straw and grain yields hill^{-1} of rice between the treatments. While the T3 treatment provided the statistically highest straw yield hill^{-1} (50.98 g hill^{-1}), the other inoculated and exclusive FYM treatments recorded statistically similar values. The grain yield per hill also followed a similar trend of variation as that of straw yield, with the T3 treatment producing the highest grain yield (36.34 g hill^{-1}) than the other treatments. The inoculated (T4, T5, T6, and T7) and the T2 treatment produced statistically similar grain yields hill^{-1}. Comparing the bio-inoculated treatments, the overall effect on grain yield, averaged over two years, could be ranked as: Can6 treatment (78% over control and 15% over T2 treatment) followed by SUND_LM2 (62% over control and 3% over T2 treatment), Can4 (59% over control and 2.4% over T2 treatment) and

Table 3
Effect of inoculation with selected diazotrophic isolates on growth and yield of rice (two years pooled data).

Treatments	Plant height (cm)	No. of effective tillers hill^{-1}	Panicle length (cm)	Thousand grains weight (g)	Straw yield (g hill^{-1})	Total grain yield (g hill^{-1})	Nitrogen uptake (mg hill^{-1})
Control (T1)	80 c	9.00 c	16.86 d	15.52 d	24.32 c	17.01 c	44.39 d
FYM@5 t ha^{-1} (T2)	89.6 b	10.00 b	21.03 bc	20.00 bc	36.15 b	26.50 b	70.90 c
NPK (60:30:30 kg ha^{-1}) (T3)	102.67 a	18.00 a	27 a	28.85 a	50.98 a	36.3367 a	96.79 a
FYM@5 t ha^{-1} + SUND_BDU1 (T4)	91.3 b	11.00 b	20.73	19.58 c	36.69 b	26.82 b	77.02 bc
FYM@5 t ha^{-1} + SUND_LM2 (T5)	91.6 b	10.00 b	21.40 bc	20.26 bc	38.10 b	27.53b	69.16 c
FYM@5 t ha^{-1} + Can4 (T6)	89.6 b	11.00 b	21.40 bc	19.83 bc	36.49b	27.15 b	73.97 bc
FYM@5 t ha^{-1} + Can6 (T7)	97.6 b	12.00 b	22.83 bc	20.86 b	38.63 b	28.00 b	82.04 b

Figures denoted by same alphabets are statistically similar at 5% probability level by DMRT.

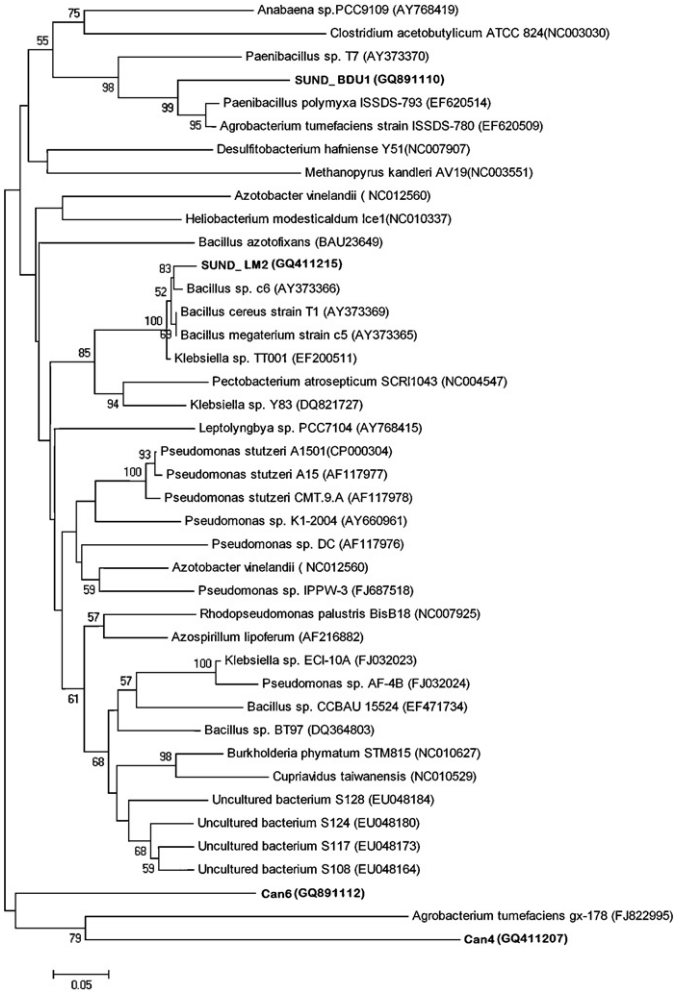

Fig. 3. Neighbor joining phylogenetic tree based on partial *nifH* sequences. Tree generated with *Anabena* sp. PCC9109 as outgroup. The database accession numbers are indicated after the bacterial names. The scale bar represents 0.05 substitutions per site. The bootstrap values are presented above the nodes (values below 50% are not shown).

SUND_BDU1 (50% over control and 1.2% over T2 treatment). The nitrogen uptake was the statistically and significantly highest with the T3 treatment (96.79 mg hill^{-1}). The other treatments followed a decreasing order of: T7 (82.04 mg hill^{-1}) > T4 (77.02 mg hill^{-1}) > T6 (73.97 mg hill^{-1}) > T2 (70.90 mg hill^{-1}) > T5 (69.16 mg hill^{-1}) > T1 (44.39 mg hill^{-1}).

The effect of inoculation on growth and yield parameters of Lady's finger cultivated during summer season following rice is presented in Table 4. The T3 treatment registered the tallest plant (89.6 cm) and the control treatment (47.3 cm), the shortest. Between the inoculated treatments, although the T7 (59.6 cm) treatment had taller plants, it was statistically similar to the T5 treatment (55.6 cm). All the inoculated treatments produced taller plants than the control treatment T1. Only the T7 and T5 treatments recorded statistically higher plant height than the T2 treatment. The number of leaves also followed the similar trend in chronological order of magnitude as that of the plant height. The statistically highest fresh fruit yield could be obtained with the T3 treatment (18.09 kg plant^{-1}). Following next in order was the T7 treatment (15.93 kg plant^{-1}). The other inoculated treatments, i.e. the T4, T5 and T6 treatments produced statistically similar fresh fruit yields, but recorded statistically higher values than the T2 and T1 treatments. The total nitrogen uptake was the highest in the T3 treatment (82.41 mg plant^{-1}). The other treatments were in the order of: T7 (69.68 mg plant^{-1}) > T6 (58.03 mg plant^{-1}) > T5 (53.44 mg plant^{-1}) > T4 (52.86 mg plant^{-1}) > T2 (49.02 mg plant^{-1}) > T1 (36.50 mg plant^{-1}).

4. Discussion

Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N-poor environments (Kennedy et al. 2004). Saline soils are typically one such environment. Hence inoculation with diazotrophic bacteria might improve crop growth and productivity in such soils. Beyond 10 dS m^{-1} EC_e of soil (equivalent to 1% NaCl solution), growing agricultural crops become impossible. Thus the organisms were screened by growing them in culture media containing 1% NaCl, so that the organisms could be used as potential microbial inoculants for agriculture in coastal saline soil. Under such conditions the isolates reported in this study showed varying levels of IAA production albeit lower than the control strain. Variation in IAA production in culture media is already reported with *Pseudomonas paucimobilis* SD13 (0.36 μg ml^{-1}) (Sarwar and Kremer, 1995), *Pseudomonas putida* (CC-FR2-4) (42.8 μg ml^{-1}) (Rekha et al., 2007) and different strains of *Acetobacter diazotrophicus* (Fuentes-Ramirez et al., 1993) producing a wide range of IAA (19–65 μg ml^{-1}). The range of IAA production in several phosphate solubilizing bacterial isolates was 57–288 μg ml^{-1} culture media (Shahab et al. 2009). Amounts of IAA produced by our isolates appear to be low compared to the previous reports. We noted that in majority of the cases the IAA production was measured in the absence of saline stress while our selection and measurement conditions necessitated the presence of 1% NaCl in culture conditions to check whether they could potentially promote plant growth under saline conditions. The IAA increases plant metabolism and hence inoculation of diazotrophic IAA producing strains in saline soil is of immense importance, because in addition to increasing the nitrogen status of soil, it may promote plant growth (Alexander 1977). The studied organisms were both diazotrophic and producer of IAA which make them good candidates for crop growth.

Fixation of atmospheric nitrogen together with solubilization of insoluble phosphate by diazotrophs is rare and to our knowledge such examples of diazotrophic phosphate solubilizer are

Table 4
Effect of inoculation with selected diazotrophic isolates on growth and yield of Lady's finger (two years pooled data).

Treatments	Plant height (cm)	Number of leaves plant^{-1}	Fresh fruit yield (kg plant^{-1})	N-uptake (mg plant^{-1})
Control (T1)	47.3 d	8 e	7.57 e	36.50 f
FYM@5 t ha^{-1} (T2)	51.3 c	12 cd	11.77 d	49.02 e
NPK (60:30:30 kg ha^{-1}) (T3)	89.6 a	21 a	18.09 a	82.41 a
FYM@5 t ha^{-1} + SUND_BDU1 (T4)	53 c	14 c	12.81 c	52.86 d
FYM@5 t ha^{-1} + SUND_LM2 (T5)	55.6 b	11 d	13.09 c	53.44 d
FYM@5 t ha^{-1} + Can4 (T6)	53 c	13 cd	12.63 c	58.03 c
FYM@5 t ha^{-1} + Can6 (T7)	56.6 b	17 b	15.93 b	69.68 b

Figures denoted by same alphabets are statistically similar at 5% probability level by DMRT.

Pantoea agglomerans (Verma et al., 2001) and *Swaminathania salitolerans* (Loganathan and Nair, 2004). Phosphate solubilizing bacteria (phosphobacteria) posses the ability to solubilize insoluble inorganic phosphate by producing acids (Alexander 1977) rendering it available to plants. However, production of phosphatase in the presence of inorganic sources of phosphorus in the culture media indicated that there could also be a possible role of phosphatase in solubilizing inorganic phosphate. Significantly higher degree of phosphate solubilization from tricalcium phosphate than aluminum phosphate in culture media could be related to the adaptation of the isolates to respective phosphates. The pH of the coastal saline soils in the Sundarbans varies from neutral to slightly alkaline. Such alkaline soils are rich in calcium phosphate, while aluminum phosphate is a major constituent of acidic soils. Since the organisms were adapted to calcium phosphate exposure in their natural habitat, their ability to solubilize calcium phosphate was probably much more than that of aluminum phosphate.

16S rRNA and *nifH* share a common evolutionary history (Hennecke et al. 1985; Ueda et al. 1995), and both are used as molecular markers in diversity studies (Widmer et al. 1999; Ohkuma et al. 1999). Demonstration of diazotrophic activity in some members of *Agrobacterium* (Kanvinde and Sastry 1990) and *Bacillus* (Ding et al. 2005) is already reported. This uncertainty in phylogenetic placement of *nifH* gene in Can4 and Can6 could be due to acquisition from other species through horizontal gene transfer and subsequent fixation in the genome through adaptive evolution. The acquisition of foreign DNA may occur through specific DNA uptake systems (Koonin et al. 2001). Lateral transfers of *nifH* among rhizobia (Haukka et al. 1998; Eardly et al. 1992), and from a common donor in the α-proteobacteria to *Azoarcus* sp., a β-proteobacteria (Hurek et al., 1997), have already been described. The idea of lateral transfer of *nifH* is further supported by the unusual position of *Frankia* and *Anabaena* within the proteobacteria clade in the *nifH* tree of Normand and Bousquet (1989). Therefore a more detailed analysis is required to understand the organization and components of nitrogen fixation machinery in the isolates described here.

Plant growth promotion by bio-inoculation with diazotrophs as well as other PGPR has been frequently reported. Tillering is an important phenological event in rice development. Similar to our findings, Tran Van et al. (2000) noticed an increase in number of effective tillers when *Burkholderia vietnamiensis* was inoculated to rice. Okon and Labandera-Gonzalez (1994), from greenhouse and field experiments around the world, also mentioned, 5–30% increase in grain yield from 70% of the inoculated trials evaluated. Such results clearly demonstrate the plant growth promotion in rice by bioinoculants. This growth promotion could be the result of simultaneous production of IAA and biological N_2 fixation by the diazotrophic inoculants (Biswas et al. 2000), or due to the contribution through nitrogen fixation only (Jha et al. 2009). Jha et al. (2009) reported the growth promoting effect of diazotrophic bacterial strains of *Herbaspirillum seropedicae*, *Glucanoacetobacter diazotrophicus*, *Azospirillum brasilense*, *Burkholderia cenocepacia* on rice. It was observed that inoculation of all the strains individually resulted in an increase in shoot length, root length, shoot dry weight, root dry weight and grain yield than the control. The inoculation of a liquid formulation of *A. brasilense* increased the number of harvested wheat grains by 6% and grain yield by 8% (Zorita and Canigia 2009).

Several field experiments were previously carried out to study the effect of diazotrophic microbial inoculants on the yield attributes of different vegetable crops. Inoculation of diazotrophic strains had a considerable impact on the growth and nutrient uptake of Chinese cabbage. Inoculation of the diazotrophic bacterial strains significantly increased the biomass of plants and also had a significant impact on the total N in plant tissues when compared to uninoculated control (Yim et al. 2009). A native strain of *Azotobacter*, was found to increase the yield of *Allium cepa* (onion) in alkaline soil by 61.9% over uninoculated control plots (Kashyap et al. 2005), which corroborates with our findings with respect to superior efficacy of a native diazotrophic strain in improving crop yield.

Unusual types of diazotrophic bacteria of diverse phylogenetic groups with strong diazotrophic potential occur in coastal saline soils. Microbial inoculants prepared out of these bacterial strains could be gainfully utilized for agriculture in coastal saline soils. In this study, four different diazotrophic bacterial strains were utilized for preparing microbial inoculants. The strains provided beneficial effect in crop production under rice–Lady's finger cropping system. The bioinoculants used in this study comprised of the isolates from coastal forest area and coastal agricultural land. The strain from the agricultural land provided significant beneficial results, in increasing crop production possibly due to its adaptation in such soil. Higher efficiency of one of the strains could be traced to its higher nitrogen fixing potential, IAA production and higher degree of mineralization of β-glycerophosphate, compared to the other strains. Considering the input cost, sustainability and eco-friendly agriculture, application of area specific bioinoculant is strongly advocated.

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