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Characterization and crop production efficiency of diazotrophic isolates from the rhizosphere of semi-arid tropical grasses of India

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ABSTRACT

The search for diverse plant growth-promoting diazotrophic bacteria is gaining momentum as efforts are made to exploit them as bioinoculants for various crops. In particular, the use of strains with multiple plant growth promoting properties would help to increase crop productivity on a sustainable basis. This study investigated the effects of plant growth promoting potential of diazotrophs isolated from rhizosphere of semi-arid tropical grasses and evaluated their inoculation effects on the growth of rice plants under *in vitro* and *in vivo* conditions. The diazotrophic isolates from grass species were characterized for nitrogenase activity by acetylene reduction assay (ARA) and 16S rRNA gene sequencing. The ARA activity of the isolates ranged from 50.83 to 172.25 nmol ethylene/mg protein/h and the putative diazotrophs from rhizosphere of grass species were identified by *nifH* gene amplification. The 16S rRNA gene sequence analysis identified the isolates as belonging to class of alpha Proteobacteria and Firmicutes. Plant growth promoting traits of all the selected diazotrophic isolates were analysed and results revealed that the diazotrophs were found to produce phytohormone, siderophores, HCN, solubilized minerals such as P, K and Zn. Diazotrophs also produced enzyme such as ACC deaminase that can modulate plant growth and development. Based on the presence of multiple plant growth promoting traits, the isolates were selected for inoculation studies. In gnotobiotic experiment, inoculation of diazotrophic isolates significantly improves the growth of rice. In the field experiment, *Serratia* sp. (CB2) and *K. pneumoniae* (CR3) treated plots, grain yields were recorded more by 31 and 28%, respectively, over yield obtained using full doses of fertilizers. This trait of improving growth parameters and yield of rice indicates that the diazotrophs isolated from grass species can be utilized as bioinoculant for rice.

1. Introduction

In the context of increasing global concern for food security and environmental quality, the use of bioinoculants like plant growth-promoting diazotrophs in agriculture is a potentially important issue for more grain yield and to less chemical inputs (Dey et al., 2004; Minorsky, 2008). The improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, where both rely on soil biological processes and soil biodiversity.

Nitrogen fixation by plant-symbiotic bacteria, an eco-friendly biological process has been effectively exploited for important leguminous crop species. Although associations of diazotrophic bacteria with non-leguminous plants such as grasses have been known for decades (Dobereiner, 1977), they have been less studied in other crop plants except for a few cases; for example, associative bacteria of some tropical species of rice and maize (Reis et al., 2000). Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N-poor environments (Cocking, 2005). A more complete understanding of the diversity and function of diazotrophic microorganisms, especially those that have clear relationships with commercially important non-leguminous plant species is of great value for research and application (Doty et al., 2009). The rhizosphere microbiology of native plants is important in view of the *in situ* conservation of the biodiversity associated with such niches to sustain delicate ecological processes in the oligotrophic ecosystem. These diazotrophs could be very useful in the formulation of new microbial inocula and could be applied most profitably to economically important non-legume crops (Cocking, 2005).

For instance, apart from its ability to convert atmospheric dinitrogen (N_2) into ammonia (NH_3) that can be used by plants, *Azospirillum* sp. also possesses an array of other plant growth

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promoting traits, such as nutrient solubilization, uptake and enhanced stress resistance (Dobbelaere et al., 2003). In this regard, N_2-fixing bacterias belonging to the genera *Azospirillum*, *Herbaspirillum*, *Burkholderia*, and *Pseudomonas* appear to be frequent colonizers of important crop plants and have been extensively studied (Mirza et al., 2006). Thus, different plant growth promoting bacteria may promote growth of crop plants by several mechanism, *viz.* by synthesizing various phytohormones such as indole 3-acetic acid (IAA), producing siderophores that can provide iron to plants, solubilizing minerals such as P, and synthesizing enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase that can modulate plant growth and development (Lucy et al., 2004). A particular plant growth promoting bacteria may enhance plant growth and development using any one or more of these mechanisms. Inoculation of plants with plant growth promoting bacteria (PGPB) not only increases plant growth but also improves total NPK uptake (Shaharoona et al., 2007).

The association of diazotrophs with rice is one alternative that has been strategically thought to replace part of the N fertilizer required by the plant and in addition, indirectly helping the plant to assess other nutrients added or naturally present in the soil. Mahadevappa and Shenoy (2000) reported that free-living heterotrophic N_2-fixers are potentially important source of N_2-fixation in rice fields, and many researchers have addressed the beneficial effects of N_2-fixing systems on rice growth using different strains under greenhouse and field conditions (Ladha and Reddy, 2003; Muthukumarasamy et al., 2007; Choudhury and Kennedy, 2004).

The rhizosphere of plant species growing profusely under stress-conditions harbors novel diazotrophs to meet their nitrogen requirement as observed in salt marsh grasses such as *Spartina alterniflora*, *Juncus roemerianus* (Bagwell and Lovell, 2000) and *Salicornia virginica* (Bagwell et al., 1998), oligotrophic habitant *Drosera villosa* (Albino et al., 2006) and desert growing *Lasiurus* grass (Chowdhury et al., 2009). Hence, it is widely accepted that the rhizosphere of any plant species is a unique niche harboring diversified bacterial and fungal communities, which serve as potential resource for bioprospecting. Accordingly, present investigation was undertaken to study diverse group of multiple plant growth promoting diazotrophs associated with semi arid tropical grasses and evaluated their inoculation effects on rice under gnotobiotic and field conditions.

2. Materials and methods

2.1. Rhizosphere soil sampling and isolation of diazotrophs

Based on their predominance in each physiographic region, a total of 10 different grass species were sampled and presented in Table 1 (Dixit et al., 2008). Plants were uprooted carefully and the soil adhering to the root was separated in a sterile petri dish and mixed thoroughly so as to make a composite sample for microbiological analysis. Soil samples collected were transported to laboratory in ice box for further analysis (Pramer and Schmidt, 1966). Diazotrophic microorganisms were isolated using serial dilution technique on four selective N-free media *viz.*, N-free malate medium (NFMM) (Piao et al., 2005), LGI-P (Reis et al., 2000), total diazotroph medium (TDM) (Dobereiner, 1989) and junior N-free bromothymol blue medium (JNFb) (Kirchhof et al., 1997). Aliquots (0.1 ml) from the serially diluted samples were added to four different media in petri plates and kept in an incubator at 30 °C. Five days after incubation, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies were picked from the petri dishes and sub-cultured to obtain pure cultures. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at −80 °C.

2.2. Authentication of diazotrophy

The bacterial isolates grown in N-free media broth for 4 days at 28 ± 2 °C were assayed for nitrogenase activity by ARA using gas chromatograph (Chemito 7610) equipped with flame ionization detector and Poropak-N column by following standard procedure as described by Park et al. (2005).

The presence of *nifH* gene was determined by amplifying the 450 bp fragment using a pair of specific degenerated primers as described by Burgmann et al. (2004). For this, total DNA of the diazotrophs was isolated using the standard protocol of hexadecyltrimethyl ammonium bromide (CTAB) method (Melody, 1997) and dissolved in distilled water for final concentration of 20 ng/μl and stored at 4 °C. The *nifH* amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) with a 25 μl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 μM of each primer (Burgmann et al., 2004), 2.5 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions: initial denaturation at 95 °C for 5 min, 35 cycles consisting of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (primer extension) and final extension at 72 °C for 5 min. The amplified products were analysed by electrophoresis in 1.5 percent agarose gels. After separation of the PCR products in agarose gel, it was viewed and photographed using InGenius gel documentation (Syngene, UK) and analysis system.

2.3. Identification of diazotrophs by 16S rRNA gene sequencing

Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2 (Weisburg et al., 1991) and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using pTZ57R/T vector supplied with TA cloning kit (Fermentas, USA)

Table 1
Details of grass species from different physiographical regions of India used for the present investigation.

Grass species	Sampling site	Latitude	Longitude	Physiographic region
Brachiaria reptans (water grass)	Barrackpur, Kolkata, West Bengal	88° 34′ 5.1″ E	22° 19′ 49.6″ N	Indo gangetic alluvial plain
Cenchrus glaucus (buffel grass)	Chadrapur, Ganjam, Orissa	88° 24′ 22.8″ E	19° 24′ 21.09″ N	Eastern ghats
Saccharum spontaneum (wild sugarcane)	Madan Mahal, Jabalpur, Madhya Pradesh	79° 40′ 50.33″ E	22° 51′ 17.03″ N	Central highlands
Panicum repens (torpedo Grass)	Maruteru, West Godaveri, Andrapradesh	80° 59′ 38.86″ E	16° 30′ 39.7″ N	Deccan plateau
Cyperus rotundus (nut grass)	Chickarasinikere, Mandya, Karnataka	77° 3′ 35.9″ E	12° 17′ 34.78″ N	Reverain land form
Dactyloctenium aegyptium (crowfoot grass)	Kasargod, Kerala	75° 7′ 59.81″ E	12° 24′ 31.4″ N	Kerala plains
Chloris barbata (finger grass)	Thavalakuppam, Pudhucherry	76° 46′ 54.7″ E	11° 23′ 12.6″ N	Coastal plains
Oryza rufipogon (wild rice)	Gudalur, Ooty, Tamil Nadu	79° 51′ 33.1″ E	11° 54′ 32.52″ N	Western ghats
Cyanodon dactylon (bermuda grass)	Navalur kutapattu, Trichy, TamilNadu	79° 46′ 34.9″ E	10° 33′ 21.32″ N	Reverain land form
Setaria verticillata (bristly foxtail)	Thirupoondi, Nagapattinam, Tamil Nadu	79° 53′ 37.6″ E	10° 46′ 25.67″ N	Coastal plains

prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST).

2.4. Determination of plant growth-promoting traits

To quantify the IAA, 1 ml of the cultures at exponential stage was inoculated in 100 ml LB medium containing filter sterilized L-tryptophan (0.01% w/v). All the flasks were wrapped with black paper to avoid photo inactivation. The flasks were incubated at room temperature for 7 days. The cell free extracts were assayed according to Gorden and Paleg (1957). ACC deaminase activity was determined by growing the cells in minimal medium with 3 mM ACC as the sole N source. Production of α-ketobutyrate as a result of enzymatic cleavage of ACC by ACC deaminase was measured at 540 nm as per the method described by Penrose and Glick (2003) and compared with a standard curve of α-ketobutyrate (Sigma–Aldrich, USA). Production of siderophores by the bacterial isolates were performed on Chrome Azurol S (CAS) agar plates and incubated for 24 h at room temperature (Schwyn and Neilands, 1987). The formation of bright yellowish fluorescent color zone by the culture in the medium indicated siderophore production. The presence of catechol type siderophore was determined by Arnow's assay (Arnow, 1937). The ability to produce cyanide (HCN) was determined as per the method of Lorck (1948). The antagonistic activity of all the diazotrophic isolates against 3 plant pathogenic organism *viz.*, *Rhizoctonia solani* (sheath blight), *Pyricularia oryzae* (blast) and *Sarocladium oryzae* (sheath rot) were evaluated as described in Dennis and Webster (1971). One microliter of each LB pure bacterial culture was inoculated in plates containing potato dextrose agar, and a 3-cm-diameter cylinder of mycelia was introduced in the plate center and incubated for 10 days at 25 °C. Percent inhibition in colony growth in plate assay was calculated by using the following formula:

$$\% \text{ Inhibition} = [(C - T)/C] \times 100$$

where, C = colony growth in control; T = colony growth in dual culture.

The mineral P- and Zn-solubilizing ability of the strains was determined on Pikovskaya's agar medium (Katznelson and Bose, 1959) amended with 0.5% tricalcium phosphate and 0.12% zinc oxide (Bunt and Rovira, 1955) as inorganic P and Zn sources, respectively. Bacterial cultures were streaked in the Aleksandrov medium (Aleksandrov et al., 1967) containing 0.25% potassium aluminum silicate as insoluble source for potassium solubilization. Bacteria having solubilization potential were identified by the appearance of a clear halo around colonies against an opaque background. The solubilizing efficiency was calculated as diameter of solubilization zone–colony diameter/colony diameter ×100.

2.5. Plant inoculation experiments

2.5.1. Gnotobiotic experiment

To study the impact of diazotrophic isolates on the growth of rice plants, dehulled rice seeds (cultivar – ADT 43) were surface sterilized by immersion in 70% ethanol for 30 s, followed by soaking in 0.2% mercuric chloride for 30 s and then washed several times with sterilized distilled water. The surface sterilized seeds were germinated aseptically in 1% sucrose agar medium. Three days old seedlings that were free of any visual bacterial and fungal contamination were used for inoculation with diazotrophic isolates. The elite multi-functional diazotrophic trains were grown in LB broth till the population reached to 10^{10} cells/ml. The cells were then harvested by centrifugation at 6000 rpm for 5 min at room temperature. The cell pellets were washed twice with 20 ml of phosphate buffer and resuspended in 1.5 ml of phosphate buffer. Seeds were treated with the selected bacterial inoculants for 15 min. Pre-germinated seeds were placed at the rate of one seed in each 200 ml culture tube containing 40 ml of N-free Fahraeus medium (Fahraeus, 1957). The seedlings were grown in a growth chamber at 27 °C (HECO plant growth chamber). Shoot length, root length and dry biomass were recorded at 21 days after sowing.

2.5.2. Field experiment

Field experiment was conducted in Wetland Farm, Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore during rabi season, 2013. The experiment was carried out in randomized block design (RBD) with three replications for each treatment. Physico-chemical properties of soil collected from the experimental field were, pH 8.17, electrical conductivity 4.32 dSm^{-1}, organic carbon 0.99%, available nitrogen 225.6 kg/ha, available phosphorus 18.6 kg/ha and available potassium 345.7 kg/ha. The elite multi-functional diazotrophic strains were grown in LB broth till the population reached to 10^{10} cells/ml. Paddy seeds were treated with the above bacterial inoculants after preparing semisolid slurry by mixing with carboxy methyl cellulose as an adhesive and shade dried for 30 min before sowing. For the observations, three hills were randomly collected at harvest from each replicated plot and the following crop measurements were made on plant height, number of effective tillers per hill, panicle length, thousand grain weight, straw yield and grain yield. The nitrogen content in the plant sample on dry weight basis was estimated by Micro Kjeldhal method as suggested by Subbiah and Asija (1956) and expressed as kg/ha.

2.6. Statistical analyses

All the data were subjected to statistical analysis with softwares, SPSS (Kirkpatrick and Feenay, 2005) and Microsoft Excel for Windows 2007 add-ins with XLSTAT Version 2010.5.05 (XLSTAT, 2010). Data was subjected to ANOVA and statistically significant differences between the treatments were analyzed using Duncan's Multiple Range Test (DMRT) at 5% level of significance. Relationships between different parameters were studied using different models which were fitted to data on nitrogenase activity, IAA and phosphorus solubilization with grain yield. Best fit models are presented in the figures.

3. Results

3.1. Soil analysis

The samples collected from different locations were analysed for soil reaction and salt concentrations. The various grass species that dominated the different soil groups are *C. glaucus* (slightly acidic), *O. rufipogon* (highly acidic), *C. dactylon* (alkaline), *B. reptans* (saline), *S. spontaneum* (saline), *P. repens* (saline), *C. barbata* (saline), *S. verticillata* (saline), *C. rotundus* (saline-sodic) and *D. aegyptium* (acid sulphate) (Table 2).

3.2. Diazotrophic bacteria isolation and Identification

Altogether sixty diazotrophic isolates were obtained by using four N-free media after 5 days of incubation. The highest number of diazotrophs were isolated from the rhizosphere soil samples of *O. rufipogon* and N-free malate medium gave the maximum amount of diazotrophic isolates (32) followed by LGI (28) medium (Table 3). Out of 60 isolates, there were about eleven unique bacterial colonies from different grass species were further

Table 2
Physio-chemical properties of rhizosphere and bulk soil samples of grass species collected from different physiographic regions.

Grass species	Source	pH	EC (dSm^{-1})	ESP %	Available nutrients (kg/ha)		
					N	P	K
B. reptans	R	7.2 (±0.18)^{i}	4.5 (±0.23)^{e}	10.5 (±0.67)^{e}	161.4 (±23.5)^{c}	27.5 (±12.6)^{g}	134.2 (±13.8)^{p}
	B	7.4 (±0.28)^{gh}	4.6 (±0.24) ^{de}	10.7 (±0.34)^{d}	150.4 (±21.5)^{f}	23.5 (±12.8)^{m}	127.2 (±12.2)^{r}
C. glaucus	R	6.2 (±0.08)^{j}	2.7 (±0.98)^{g}	4.2 (±0.34)^{l}	128.8 (±13.1)^{p}	40.5 (±12.7)^{a}	295.3 (±24.1)^{a}
	B	6.2 (±0.08)^{j}	2.7 (±0.98)^{g}	4.2 (±0.34)^{l}	126.5 (±12.5)^{q}	39.5 (±13.7)^{b}	285.3 (±23.1)^{b}
S. spontaneum	R	8.2 (±0.28)^{d}	4.5 (±0.56)^{e}	8.1 (±0.45)^{i}	184.7 (±12.8)^{a}	39.3 (±14.7)^{c}	230.5 (±45.7)^{e}
	B	8.5 (±0.26)^{c}	4.6 (±0.36)^{de}	8.3 (±0.45)^{gh}	180.7 (±23.8)^{b}	34.2 (±12.7)^{d}	213.5 (±47.9)^{g}
P. repens	R	7.3 ±0.23)^{hi}	4.7 (±0.14)^{d}	8.5 (±0.42)^{f}	119.8 (±15.9)^{r}	19.5 (±16.9)^{p}	117.4 (±13.5)^{s}
	B	7.5 ±0.45)^{fg}	4.5 (±0.13)^{e}	8.5 (±0.23)^{f}	103.8 (±12.9)^{s}	14.5 (±12.9)^{s}	109.4 (±11.4)^{t}
C. rotundus	R	8.7 (±0.22)^{ab}	4.2 (±0.12)^{f}	18.5 (±0.41)^{c}	156.8 (±23.6)^{e}	29.0 (±18.9)^{e}	159.5 (±15.2)^{m}
	B	8.8 (±0.32)^{a}	4.3 (±0.24)^{f}	18.5 (±0.54)^{c}	128.8 (±16.8)^{p}	27.0 (±17.9)^{h}	134.5 (±12.1)^{q}
D. aegyptium	R	3.8 (±0.26)^{m}	0.8 (±0.06)^{h}	1.2 (±0.78)^{0}	140.5(±16.8)^{k}	25.0 (±15.9)^{j}	188.7 (±14.4)^{i}
	B	3.9 (±0.26)^{m}	0.8 (±0.07)^{h}	1.3 (±0.78)^{0}	138.6 (±1.11)^{l}	23.4 (±16.7)^{m}	180.7 (±12.4)^{j}
C. barbata	R	7.7 (±0.45)^{e}	6.3 (±0.24)^{b}	7.5 (±0.45)^{k}	149.5 (±23.6)^{h}	28.0 (±16.8)^{f}	214.9 (±22.7)^{f}
	B	7.7 (±0.45)^{e}	6.6 (±0.24)^{a}	7.7 (±0.45)^{j}	147.5 (±46.6)^{j}	22.6 (±16.8)^{n}	213.9 (±21.5)^{g}
O. rufipogon	R	5.3 (±0.67)^{l}	0.72 (±0.04)^{h}	1.5 (±0.35)^{n}	132.4 (±12.7)^{n}	26.8 (±14.4)^{i}	170.4 (±23.8)^{k}
	B	5.6 (±0.57)^{k}	0.77 (±0.04)^{h}	1.8 (±0.38)^{m}	130.4 (±10.7)^{0}	24.8 (±12.6)^{l}	168.3 (±21.9)^{l}
C. dactylon	R	8.6 (±0.18)^{bc}	0.18 (±0.06)^{i}	25.0 (±0.13)^{b}	152.4 (±16.8)^{f}	19.3 (±12.6)^{q}	278.5 (±13.1)^{c}
	B	8.8 (±0.18)^{a}	0.20 (±0.08)^{i}	27.0 (±0.34)^{a}	160.4 (±16.8)^{d}	18.3 (±10.4)^{r}	268.9 (±12.1)^{d}
S. verticillata	R	7.5 (±0.89)^{fg}	5.8 (±0.67)^{c}	8.2 (±0.67)^{hi}	148.3 (±18.9)^{i}	25.5 (±32.6)^{i}	154.6 (±31.8)^{n}
	B	7.6 (±0.95)^{ef}	5.7 (±0.78)^{c}	8.4 (±0.79)^{fg}	133.3 (±18.9)^{m}	21.5 (±32.6)^{0}	143.5 (±32.8)^{0}

Values are mean (±SE) ($n = 3$) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).
R: rhizosphere; B: bulk soil; EC: electrical conductivity; ESP: exchangeable sodium percentage.

Table 3
Selected diazotrophic isolates from rhizosphere of grass species collected from different physiographic regions

Grass species	Isolate code	Isolation media	Number of diazotrophs	Total number of diazotrophs
B. reptans	BR	NFMM	3	5^{c}
		LGI	1	
		JNFb	1	
C. glaucus	CG	NFMM	5	7^{b}
		LGI	2	
S. spontaneum	SS	NFMM	3	5^{c}
		LGI	1	
		TDM	1	
P. repens	PR	NFMM	2	5^{c}
		LGI	2	
		JNFb	1	
C. rotundus	CR	NFMM	3	6^{bc}
		LGI	3	
D. aegyptium	DA	JNFb	1	2^{d}
		TDM	1	
C. barbata	CB	NFMM	3	7^{b}
		LGI	2	
		JNFb	2	
O. rufipogon	OR	NFMM	5	10^{a}
		LGI	3	
		JNFb	2	
C. dactylon	CD	NFMM	4	7^{b}
		LGI	3	
S. verticillata	SV	NFMM	4	6^{bc}
		TDM	2	

Values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).
NFMM: N free malate medium; Nfb: N free bromothymol blue medium; JNFb: junior N free bromothymol blue medium; TDM: total diazotroph medium.

Table 4
Isolation media, colony characteristics and nitrogenase activity of the diazotrophic isolates.

Isolate code	Closest relative in database	GenBank accession no.	Rhizosphere soil of grass species	Isolation media[a]	General properties		ARA activity[b]	*nifH* gene
					Colony color	Gram's reaction		
BR1	*Enterobacter* sp.	KF906826	*B. repens*	NFMM	Creamy white	–	84.45 (±3.98)^{g}	+
CG1	*Enterobacter* sp.	KF906827	*C. glaucus*	LGI	Creamy white	–	107.3 (±14.35)^{d}	+
CG3	*Enterobacter* sp.	KF906828	*C. glaucus*	JNFb	Cream	–	99.45 (±6.41)^{e}	+
CG5	*Bacillus* sp.	KF906830	*C. glaucus*	NFMM	Creamy white	+	120.5 (±21.65)^{c}	+
CR3	*Klebsiella pneumoniae*	KF906829	*C. rotundus*	NFMM	Dull white	–	151.58 (±16.27)^{b}	+
CB2	*Serratia* sp.	KF906831	*C. barbata*	JNFb	White	–	172.25 (±13.95)^{a}	+
OR3	*Serratia* sp.	KF906832	*O. rufipogon*	NFMM	Creamy white	–	99.58 (±4.89)^{e}	+
OR5	*Staphylococcus saprophyticus*	KF906833	*O. rufipogon*	JNFb	Cream	+	50.83 (±3.28)^{i}	–
OR7	*Klebsiella* sp.	KF906834	*O. rufipogon*	LGI	Yellow	–	61.57 (±2.57)^{h}	+
CD1	*Serratia marcescens*	KF906835	*C. dactylon*	NFMM	Orange	–	89.20 (±5.43)^{f}	+
SV1	*Klebsiella pneumoniae*	KF906836	*S. vetricillata*	NFMM	Dull white	–	108.4 (±8.45)^{d}	+

Values are mean (± SE) ($n = 3$) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).
The isolates were identified by 16S rRNA gene sequence analysis. Values are mean ± standard errors of three replicates.
[a] Description was given in the materials and methods section.
[b] nmol ethylene/h/mg protein/h.

reconfirmed as putative diazotrophs by nitrogenase activity and PCR. Based on such data, eleven isolates were selected for further study.

The nitrogenase ranged from 50.83 to 172.25 nmol ethylene/mg protein/h. The highest nitrogenase activity was exhibited by isolate CB2 (172.25 ± 13.95 nmol ethylene/mg protein/h). The authentication of these isolates for diazotrophy was performed by detection of partial *nifH* by PCR. The degenerated primers amplifying the partial *nifH* gene of about 450 bp were used for authentication and the results confirmed that all but one were able to amplify the partial *nifH* gene. The selected diazotrophic bacterial colonies were characterized by morphology and Gram reaction. All the authenticated 11 diazotrophic isolates were further phylogenetically identified using 16S rRNA gene sequence homology revealed the presence of diversity of Gamma proteobacteria (82%) and Firmicutes (18%) (Table 4).

3.3. Plant growth-promoting traits

Apart from nitrogen fixation, the diazotrophic bacterial strains used in the present study possess different traits related to plant growth promoting, which are described in Table 5. All the diazotrophic isolates gave positive result with regard to IAA production and the maximum amount of IAA was produced by *Serratia* sp. CB2 (36.8 ± 1.24 μg/mg protein) followed by *Serratia* sp. OR3 (30.8 ± 1.14 μg/mg protein). Six isolates were able to produce ACC deaminase, where the isolate *Enterobacter* sp. CG1 registered the highest activity of 86.1 ± 3.56 nmol α-ketobutyrate/mg protein/h. All the isolates were able to produce siderophore and the maximum siderophore production was recorded in *Serratia* sp. CB2 (66.3 ± 3.21 μg/mg protein). Seven isolates were reported positive results for HCN production which was further quantified. The highest hydrogen cyanide production was found to be 68.7 ± 1.13 of μg/mg protein in the strain *S. marcescens* CD1.

Diazotrophic isolates exhibited significant growth-inhibitory activity against a range of phytopathogenic fungi for rice such as, *viz. R. solani*, *P. oryzae* and *S. oryzae*. Preliminary screening of antagonistic activity of all the isolates confirmed that out of 11 isolates, 2 isolates (*Serratia* sp. CB2 and *Serratia* sp. OR3) had antagonistic potential against all the three pathogens. However, the strain *S. saprophyticus* OR5 does not show any biocontrol activity against the tested pathogens. The results of the present study on mineral solubilization have revealed that the *Serratia* strains, CB2 and OR3 were found strongly solubilize all three minerals. *S. marcescens* CD1 poorly solubilized inorganic P and K, whereas *K. pneumoniae* SV1 poorly solubilized inorganic Zn.

Table 5
Plant growth-promoting characteristics of the diazotrophic isolates.

Isolate	[a]IAA	[b]acc deaminase	[a]Siderophore	[a]HCN	Antagonistic activity	Mineral solubilization efficiency%		
						P	K	Zn
Enterobacter sp. BR1	12.3 (±0.27)^{j}	ND	20.9 (±1.15)^{f}	ND	*Rs*	150 (±11.19)^{def}	ND	ND
Enterobacter sp. CG1	19.6 (±1.54)^{f}	86.1 (±3.56)^{a}	13.5 (±1.18)^{g}	30.6 (±1.56)^{g}	*Rs*,*Po*	125 (±11.29)^{fg}	ND	200 (±13.16)^{bc}
Enterobacter sp. CG3	17.7 (±1.52)^{h}	ND	22.4 (±1.10)^{e}	35.4 (±1.24)^{e}	*Rs*	100 (±13.19)^{gh}	ND	ND
Bacillus sp. CG5	21.6 (±2.16)^{e}	74.3 (±7.98)^{c}	38.4 (±1.19)^{c}	44.5 (±1.37)^{c}	*Rs*, *Po*	150 (±12.10)^{def}	ND	225 (±12.15)^{ab}
K. pneumoniae CR3	23.4 (±0.15)^{c}	45.6 (±7.98)^{d}	49.3 (±2.27)^{b}	31.4 (±1.44)^{f}	*Rs*	200 (±12.64)^{bc}	ND	ND
Serratia sp. CB2	36.8 (±1.24)^{a}	84.9 (±4.96)^{ab}	66.3 (±3.21)^{a}	62.4 (±1.58)^{b}	*Rs*, *Po*, *So*	250 (±13.53)^{a}	200 (±13.63)^{a}	250 (±13.11)^{a}
Serratia sp. OR3	30.8 (±1.14)^{b}	76.8 (±2.98)^{c}	23.5 (±2.11)^{d}	40.5 (±1.19)^{d}	*Rs*, *Po*, *So*	225 (±12.16)^{ab}	150 (±13.18)^{ab}	200 (±13.16)^{bc}
S. saprophyticus OR5	16.5 (±1.47)^{i}	ND	20.7 (±2.26)^{f}	ND	ND	75 (±1.14)^{h}	ND	ND
Klebsiella sp. OR7	22.7 (±1.25)^{d}	ND	13.7 (±1.13)^{g}	ND	*Rs*	200 (±2.64)^{bc}	ND	ND
S. marcescens CD1	18.6 (±1.26)^{g}	85.3 (±6.78)^{ab}	22.4 (±1.24)^{e}	68.7 (±1.13)^{a}	*Rs*, *Po*	60 (±1.14)^{i}	100 (±12.17)^{b}	167 (±12.24)^{cd}
K. pneumoniae SV1	11.3 (±1.22)^{k}	ND	9.8 (±1.10)^{h}	ND	*Rs*, *So*	167 (±12.63)^{de}	ND	125 (±11.64)^{e}

Values are mean (± SE) ($n = 3$) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).
ND: not detected.
Rs: Rhizoctonia solani; *Po*: *Pyricularia oryzae*; *So: Sarocladium oryzae.*
[a] μg/mg protein.
[b] nmol α-ketobutyrate/mg protein/h.

Table 6
Effect of diazotrophic bacterial isolates on biometric characteristics of rice seedlings.

Isolate	Shoot length (cm)	Root length (cm)	Plant dry matter (mg)
Enterobacter sp. BR1	13.2 (±1.23) h	6.7 (±0.55)^{g}	41.4 (±0.12)^{e}
Enterobacter sp. CG1	13.9 (±1.09)^{f}	6.9 (±0.34)^{f}	46.7 (±0.12)^{b}
Enterobacter sp. CG3	15.6 (±1.49) d	6.5 (±0.50)^{h}	41.3 (±0.22)^{e}
Bacillus sp. CG5	14.8 (±1.39)^{e}	7.2 (±0.65)^{e}	34.0 (±0.23)^{i}
K. pneumoniae CR3	16.5 (±0.73)^{c}	8.5 (±0.38)^{b}	43.8 (±0.12)^{d}
Serratia sp. CB2	18.8 (±1.42)^{a}	7.9 (±0.71)^{d}	50.0 (±0.12)^{a}
Serratia sp. OR3	17.2 (±0.14)^{b}	9.6 (±0.66)^{a}	45.2 (±0.02)^{c}
S. saprophyticus OR5	14.5(±0.45)^{f}	6.7 (±0.54)^{g}	35.1 (±0.36)^{h}
Klebsiella sp. OR7	13.6(±0.49)^{g}	6.9 (±0.54)^{f}	36.6 (±0.35)^{g}
S. marcescens CD1	13.5 (±1.31)^{g}	8.3 (±0.66)^{c}	46.8 (±0.15)^{b}
K. pneumoniae SV1	16.3(±1.46)^{c}	6.4 (±0.34)^{h}	37.3 (±0.36)^{f}
A. lipoferum Az [a]204	12.8 (±0.85)^{i}	6.8 (±0.42)^{fg}	30.0 (±0.23)^{j}
Control	12.5 (±1.22)^{i}	5.8 (±0.59)^{i}	23.0 (±0.21)^{k}

Values are mean (± standard error) ($n = 3$) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).
[a] Standard culture.

3.4. Plant inoculation experiments

In vitro assays were performed in order to evaluate the performance of 11 multiple growth promoting diazotrophic isolates, rice seedlings were raised along with commercial strain (*Azospirillum lipoferum* – Az 204) as standard. Seed inoculation with the test strains enhanced the growth and yield in almost all the treated plants in comparison to uninoculated control (Table 6). Production of maximum shoot length and plant dry matter was observed for the plant treated with *Serratia* sp. CB2 (18.8 ± 1.42 cm and 50.0 ± 0.12 mg). While, higher root length was recorded in *Serratia* sp. OR3 (9.6 ± 0.66 cm).

On the basis of multiple plant growth promoting properties, the diazotrophic strains were again used to evaluate the efficiency on the growth and yield of rice (cultivar – ADT 43) under field conditions. Application of diazotrophic isolates had a marked influence on growth and yield of rice. From the results, taller plants were observed in *Enterobacter* sp. CG1 (123.08 ± 4.78 cm) and maximum number of effective tillers recorded in *Serratia* sp. CB2 (20.8 ± 0.81) inoculated plots. Application of diazotrophic isolates had significant influence on the grain yield and the treatment *Serratia* sp. OR3 recorded higher grain yield (4997 ± 344.75 kg/ha) whereas, *Serratia* sp. CB2 showed the higher straw yield (3986 ± 154.74 kg/ha) (Table 7). With the exception of *K. pneumoniae* SV1 all of the strains increased total N content of plants compared with full recommended dose of fertilizer treatment (Table 7).

3.5. Regression analysis

Analysis revealed that there is a strong relationship that exists between grain yield and other factors like nitrogenase activity, IAA and phosphorus solubilization. They are all positively correlated with grain yield. Among these, nitrogenase activity and phosphorus solubilization show positive quadratic relationship with grain yield while IAA shows exponential relationship with grain yield (Fig. 1).

4. Discussion

Enrichment of soil with diazotrophs possessing useful plant growth-promoting (PGP) traits aids in the reduction of nitrogenous fertilizer requirement and thereby the fertilization costs as well as minimizing the risk of nitrate pollution. Plant-associated bacteria play key roles in their host's adapting to changing environments in various ecosystems. These interactions between plants and beneficial bacteria can significantly affect the general plant health and soil quality. In legumes, plant specific enrichment of microflora in the rhizosphere milieu has been exploited under nitrogen limiting conditions. Likewise, non-leguminous crops select specific bacterial groups in the rhizosphere (Lemanceau et al., 1995). Studies predict the possible role of diazotrophic bacteria in maintaining soil fertility and N input in disturbed ecosystems (Barraquio et al., 2000).

Table 7
Effect of inoculation of diazotrophic isolates on plant height (cm) of rice (cultivar-ADT43).

Isolate	Plant height (cm)	No. of effective tillers	Panicle length (cm)	Thousand grain wt (g)	Grain yield (kg/ha)	Straw yield (kg/ha)	Nitrogen uptake (kg/ha)
Enterobacter sp. BR1	110.09 (±5.75)^{h}	12.4 (±0.78)^{j}	20.4 (±0.67)^{a}	15.4 (±2.73)^{l}	3204 (±124.23)^{i}	3125 (±124.78)^{l}	55.65 (±5.34)^{g}
Enterobacter sp. CG1	123.08 (±4.78)^{a}	16.8 (±1.34)^{bc}	26.5 (±2.11)^{b}	18.9 (±1.50)^{b}	3456 (±275.08)^{h}	3874 (±308.32)^{e}	63.40 (±5.26)^{d}
Enterobacter sp. CG3	117.06 (±4.34)^{d}	10.04 (±1.02)^{k}	18.4 (±0.78)^{a}	19.3 (±1.23)^{e}	3450 (±114.78)^{h}	3453 (±174.23)^{h}	55.34 (±5.05)^{g}
Bacillus sp. CG5	113.02 (±3.45)^{f}	18.6 (±1.11)^{d}	23.7 (±1.41)^{g}	19.3 (±1.97)^{e}	3897 (±232.25)^{e}	3890 (±231.82)^{d}	60.27 (±3.59)^{e}
K. pneumoniae CR3	103.96 (±2.56)^{j}	18.7 (±2.03)^{cd}	26.3 (±2.86)^{c}	18.1 (±0.70)^{ij}	4967 (±192.81)^{b}	3906 (±425.01)^{c}	72.56 (±5.01)^{b}
Serratia sp. CB2	118.84 (±7.89)^{c}	20.8 (±0.81)^{a}	27.9 (±1.08)^{a}	20.6 (±2.30) ^{b}	4997 (±344.75)^{a}	3986 (±154.74)^{a}	78.45 (±3.04)^{a}
Serratia sp. OR3	112.76 (±8.97)^{g}	19.8 (±1.37)^{b}	27.7 (±1.92)^{a}	29.4 (±1.23)^{a}	4012 (±436.60)^{d}	3945 (±272.19)^{b}	56.30(±3.53)^{f}
S. saprophyticus OR5	107.02 (±3.58)^{i}	13.04 (±0.23)^{a}	16.4 (±0.98)^{m}	20.2 (±1.26)^{c}	3206 (±312.23)^{i}	3120 (±234.71)^{l}	54.40 (±5.57)^{h}
Klebsiella sp. OR7	120.05 (±4.78)^{b}	14.06 (±0.34)^{h}	17.9 (±2.13)^{l}	18.4 (±1.28)^{i}	3720 (±423.78)^{f}	3217 (±212.45)^{j}	68.47 (±7.13)^{c}
S. marcescens CD1	114.94 (±6.78)^{e}	18.8 (±1.18)^{c}	25.4 (±1.59)^{d}	19.7 (±1.03)^{d}	4645 (±290.96)^{c}	3784 (±236.99)^{f}	68.24 (±7.42)^{c}
K. pneumoniae SV1	121.03 (±4.78)^{b}	14.4 (±0.78)^{g}	20.04 (±1.23)^{a}	18.4 (±1.36)^{i}	3120 (±415.23)^{k}	3145 (±128.24)^{k}	48.27 (±2.99)^{j}
A. lipoferum Az [a]204	110.87 (±2.56)^{h}	14.3 (±1.05)^{g}	25.8 (±1.93)^{d}	19.1 (±1.30)^{f}	3134 (±129.23)^{j}	3445 (±257.97)^{i}	52.68 (±3.94)^{i}
100% RDF	109.63 (±8.90)^{i}	18.0 (±1.24)^{e}	24.6 (±1.70)^{f}	18.8 (±0.09)^{h}	3789 (±122.68)^{f}	3598 (±275.85)^{g}	56.96 (±3.93)
Control (absolute control)	101.82 (±5.78)^{k}	8.0 (±0.31)^{l}	23.4 (±0.91)^{g}	16.8 (±0.65)^{k}	3034 (±118.56)^{l}	3016 (±126.43)^{m}	40.04 (±1.55)

Values are mean (±SE) ($n = 3$) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).
RDF: recommended dose of fertilizer.
[a] Standard bioinoculant.

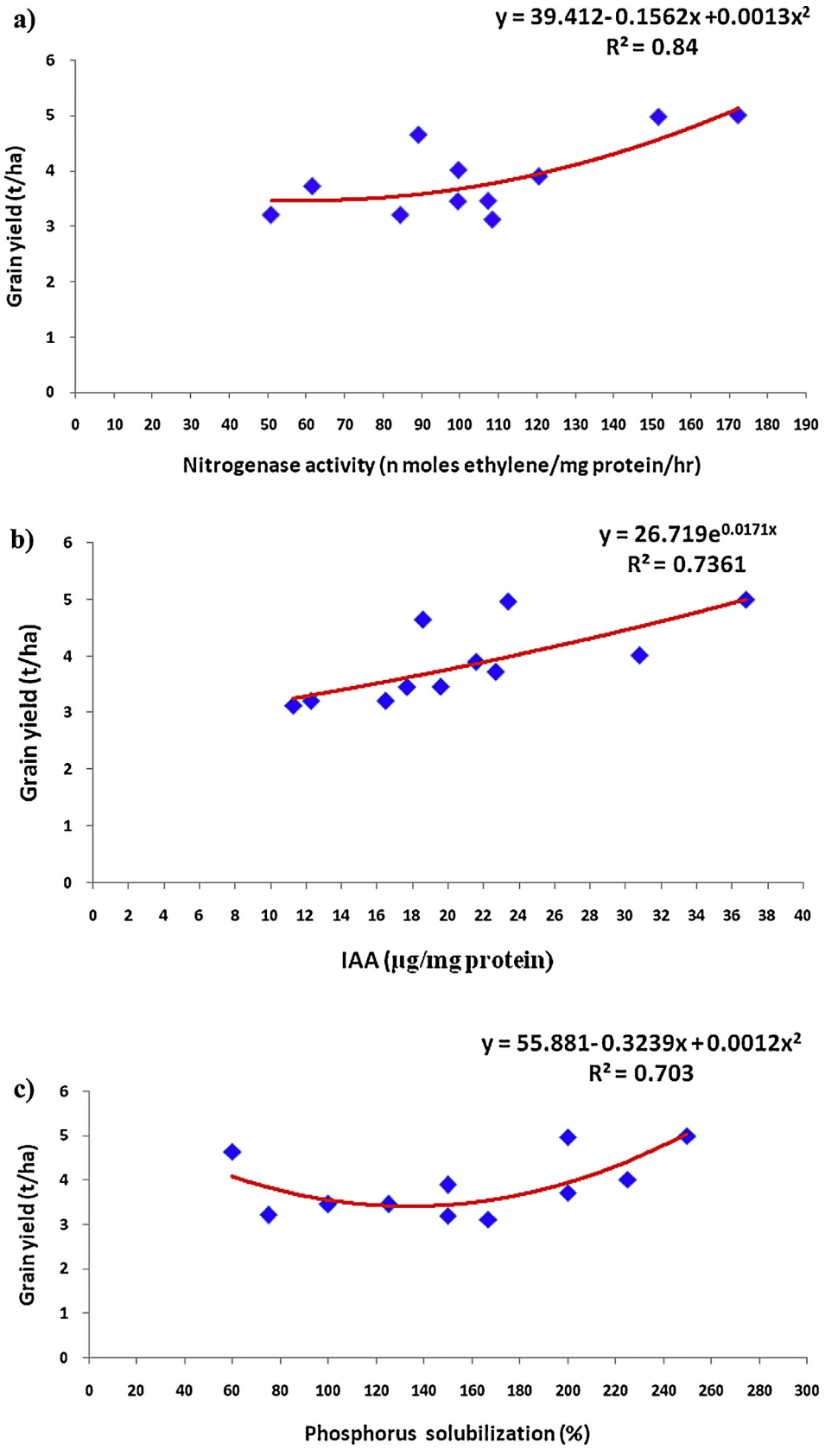

Fig. 1. Relationship of (a) nitrogenase activity (b) IAA and (c) phosphorus solubilization with grain yield of rice.

4.1. Diazotrophic diversity in grass species

Plate count analysis revealed that N-free malate medium obtained more number of diazotrophic isolates as reported by Sgroy et al. (2009). Microbes that grow in nitrogen-free medium were found to be negative in the acetylene reduction assay which also established in our study. A similar result was also found in the study of Ribbe et al. (1997).

Interestingly, in the grass rhizosphere, Enterobacteriaceae family accounts for 82% of the total diazotrophs and the members of enterobateriales are known N_2-fixers. *Enterobacter* has been identified as endophytes of several plants such as *Citrus sinensis*, soybean, sweet potato and maize (Araujo et al., 2001; Zinniel et al., 2002; Kuklinsky-Sobra et al., 2004). Gyaneshwar et al. (2001) showed that the presence of *nifH* genes was confirmed by amplification in endophytic diazotrophic strain *Serratia marcescens* isolated from rice. Iniquez et al. (2004) demonstrated and confirmed the nitrogen fixing activity of *K. pneumoniae* in wheat. The nitrogen fixing activity of *K. pneumoniae* isolates are again confirmed by our work. Diverse species of *Serratia* have been isolated from cotton and sweet corn (McInroy and Kloepper, 1995), rice rhizosphere (Rosales et al., 1993) and rice seed (Mukhopadhyay et al., 1996).

4.2. Multifaceted plant growth promoting activities

There has been increasing evidence that apart from Nitrogen fixation, synthesis and export of phytohormones by the N_2-fixing bacteria may play an important role in plant growth promotion. Our findings agree with previous reports that most of the plant associated bacteria were able to produce a variety of plant growth promoting substances in considerable amounts apart from diazotrophy. Venieraki et al. (2011) reported all the 11 strains isolated from salt marsh grass exhibited both diazotrophic and IAA production to the tune of 18.4 ± 5.4 to 194.8 ± 17.1 μg IAA/mg protein. Muthukumarasamy et al. (2007) and Ahmad et al. (2008) reported that many strains of PGPR isolated from rhizosphere soils, rhizoplane or from inside plant tissues of Gramineae plants found to have members of the Enterobacteriaceae isolated from selected grass species were competent plant growth promoting traits, with the ability to fix nitrogen, produce IAA and mineralize insoluble plant nutrients. They have earlier been also shown to be potent biological control agents against fungal diseases. Besides N fixation, the production of IAA and related compounds by *K. pneumoniae* in culture media supplemented with tryptophan was reported in our results in accordance with findings of El-Khawas and Adachi (1999).

In addition, phytohormone production, mineral solubilizing and biocontrol properties imparted by different strains of *Bacillus* sp. have also been confirmed by our work. Similarly, plant growth promoting traits of *Bacillus* sp. are widely reported by several workers (Timmusk et al., 2005; Canbolat et al., 2006; Kloepper et al., 2004; McSpadden-Gardener, 2004).

The role of ACC deaminase in decreasing ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been documented as one of the major mechanisms of PGPR in promoting root and plant growth (Hameeda et al., 2008). In nature, ACC deaminase has been commonly found in soil bacteria that colonize plant roots (Glick et al., 1999). The distribution of ACC deaminase activity is common among plant growth promoting bacterial groups. In the present study, 54% of diazotrophs showed the ACC deaminase activity. Bacteria containing ACC deaminase bind themselves to roots and/or seed coats and stimulate root elongation by lowering the ethylene level in plants. Earlier, Glick (1995) proposed that certain PGPR can regulate the production of ethylene in developing seedlings through the action of ACC-deaminase on ACC, the immediate precursor of ethylene in higher plants, hence consequently enhance root growth.

Bacterial siderophores are an important class of compounds that enhance plant growth and protects the plant health by binding to available iron (Fe^{3+}) in soils. Interestingly in this study, all the rhizospheric isolates are able to produce siderophore. Islam et al. (2009) found that 47.1% of the diazotrophic strains isolated from paddy fields are producing siderophore. However, these strains are restricted to *Serratia*, *Burkholderia* and *Herbaspirillum*. Shahi et al. (2011) reported that 21 out of 114 isolates showed multiple growth promoting activity including production of siderophores. HCN production is a common trait within the group of *Pseudomonas* present in the rhizosphere. To date many different bacterial genera have shown to be capable of producing HCN including species of *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas* and *Rhizobium* (Ahmad et al., 2008). Increase of plant growth includes a variety of mechanisms by which the bacteria prevent phytopathogens from inhibiting plant growth and development. This mechanism of plant growth promotion so far has been treated in a step motherly way as far as diazotrophs are concerned. Results of the present study on antagonistic activity of diazotrophic isolates showed that, 18% of diazotrophic isolates are antagonistic to all three pathogens (*P. oryzae, R. solani, S. oryzae*). Jaiganesh et al. (2007) found that *S. marcescens* appears to be an ideal agent for the control of *P. oryzae*, because it produces chitinolytic enzymes which cause degradation of the fungal cell walls, induction of plant defense reaction and certain antifungal low molecular weight molecules. Moreover, much evidence has indicated a potential role of such species in plant growth promotion properties as fungal biocontrol (Press et al., 1997). Similar to our findings many strains of *Bacillus* have also been widely used as microbial inoculum for improving plant growth and/or for biocontrol of pathogens in trials with wheat, spinach, strawberry and tomato (Herman et al., 2008).

Phosphorus, potassium and zinc are important plant nutrients and the beneficial role of plant growth promoting bacteria in maintaining adequate levels of these mineral nutrients in crop production has been previously reported (Rodríguez and Fraga, 1999).

Bacillus sp. has the P-solubilization capacity and could be used as inoculants and plant growth promoters to increase P-uptake by plants, as suggested by Park et al. (2005). Furthermore, the phosphate solubilization property and the presence of the nitrogen fixing genes in *S. marcescens* was demonstrated by Islam et al. (2009). A positive correlation between the potential for P and Zn solubilization has been reported (Wani et al., 2007). In the same way strains of *K. pneumoniae* (CR2), *Serratia* sp. (CB2), *Serratia* sp. (OR3), *S. marcescens* (CD1), were able to solubilize all three tested minerals in the present investigation.

4.3. Plant inoculation effects

In the present study, the dehulled and surface sterilized rice seeds treated with diverse diazotrophic strains showed the increase in root length as well as shoot length, respectively when compared with control. Islam et al. (2009) reported that inoculation of rice with free-living diazotrophic bacteria remarkably increased plant height and dry biomass production under greenhouse conditions. George et al. (2013) found a significant increase in growth and nutrient uptake accompanied with higher populations of plant beneficial microorganisms in their rhizosphere recorded on inoculation with *Serratia* sp. and *Klebsiella* sp.

In the field trial, it was observed that inoculation with diazotroph increased the rice plant height through nitrogen fixation and phytohormone production as reported by Keyeo et al. (2011). Tillering is an important phenological event in rice

development. Tran Van et al. (2000) noticed an increase in number of effective tillers when *Burkholderia vietnamiensis* was inoculated to rice. Inoculation of *Serratia* sp. CB2, *K. pnemoniae* CR3 and *S. marcescens* CD1 increased the yield by 22%–31% when compared with 100% recommended dose of fertilizer. This increase in yield could be the result of production of growth hormones, solubilization of minerals and biological N_2 fixation and biocontrol activity of diazotrophic inoculants. Verma et al. (2001) mentioned that most of the soils under rice cultivation contain insoluble phosphates. Therefore, the ability of rhizobacteria to solubilize precipitated phosphates and enhances phosphate availability to rice represents a possible mechanism of plant growth promotion under field conditions. In our study the *S. marcescens* (CD1) showed maximum solubilization of phosphorus that might have helped the crop growth. Similar to our results, George et al. (2013) mentioned that *S. marcescens* exhibited nitrogen-fixation potential, phosphate solubilization, ammonification, and production of indole acetic acid, 1-aminocyclopropane-1-carboxylate-deaminase activity, chitinase activity, siderophore production and antibiotics. In addition, seed bacterization with *S. marcescens* increased the growth parameters of test plants such as paddy and cowpea over uninoculated control in green house assay. Okon and Labandera-Gonzalez (1994) reported 3–32% increase in grain yield from 70% diazotrophs of the inoculated trials. Such results clearly demonstrate the plant growth promotion in rice by bioinoculants. Yim et al. (2009) reported inoculation of the diazotrophic bacterial strains significantly increased the biomass of plants and also 37% increase on the total N content in plant tissues when compared to uninoculated control as found in our study. Similarly Deb Roy et al. (2009) showed that seed inoculation of diazotrophs on rice have improved growth, nitrogen content, grain weight and yield of crops. Inoculation of *K. pnemoniae* (CR3) and *Serratia* sp.CB2 strain stimulated the plant growth. Our findings indicate that most of the selected strains possess multiple plant growth promoting properties that significantly improve the growth of the rice when tested under field conditions. In the "additive hypothesis", it was suggested that multiple mechanisms, such as dinitrogen fixation, phosphate solubilization, and ACC deaminase activity, together with IAA biosynthesis, are responsible for the observed plant growth promotion and yield increase (Bashan and Holguin, 1997). Inoculation of plants with plant growth promoting bacteria not only increases plant growth but also improves nitrogen uptake (Shaharoona et al., 2007; Wu et al., 2005).

The diazotrophic strains used in the present study, which were able to produce IAA and siderophores, synthesize ACC deaminase, and solubilize P, K and Zn, enhance the growth of rice under controlled as well as in field conditions. The positive results on specific plant growth promoting traits of diverse diazotrophs found in the present experiment suggests that these particular organisms can promote plant growth by more than one mechanism and that these traits could be better exploited as bioinoculants.

Free-living diazotrophs could be very useful in the formulation of new microbial inocula and could be applied profitably to non-legume crops. Besides exploring the potential for BNF and other promising plant growth promoting functions carried out by free-living diazotrophs, it is also important to ensure that the bacteria are well adapted to environmental conditions before they are utilized as inoculant strains.

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