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Diazotrophic Endophytes from the Weedy Grasses of Different Physiographic Regions of Southern India

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In the present investigation, a total of thirty two endophytic diazotrophs were isolated from rhizosphere weedy grasses of different physiographic regions of southern India. Among the diazotrophic isolates seven were screened by nitrogenase activity and higher activity of 166.25 ±13.95 n moles ethylene/mg protein /h exhibited by the isolate *Bacillus* sp.CBE9. All but one recorded the presence of *nif* gene by PCR using universal primers. The 16S rRNA gene sequence homology revealed the presence of diversity of g Proteobacteria and Firmicutes. Plant growth promoting traits of all the selected diazotrophic isolates were analysed and results revealed that diazotrophs were found to produce phytohormone, siderophores, HCN, solubilize minerals such as P, K, Zn and synthesize enzymes such as ACC deaminase that can modulate plant growth and development. Additionally, diazotrophic strains showed tolerance to various abiotic stresses such as heavy metals, salt, temperature and drought. In the plant inoculation study, the dehulled and surface sterilized seeds treated with diverse diazotrophic strains showed a 41% and 58% increase in root length as well as shoot length respectively when compare with control. The endophytic colonization in the rice seedlings (roots and culm) were examined by SEM analysis. Results indicated that endophytic bacteria were preferentially colonized in the rhizoplane and inner side of rice roots.

Key words: Endophytes, Nitrogen fixation, Plant growth promoting rhizobacteria, Scanning electron microscopy.

Most plants in their native environments depend on interactions with microorganisms for their existence. Endophytic bacteria ubiquitously inhabit most plant species and have been isolated from a variety of plants. 'Endophyte' is derived from the Greek 'endon'(within) and 'phyte' (plant), and until recently this term had usually been applied to fungi (Carroll, 1988 and Clay, 1988), including the mycorrhizal fungi (O'Dell and Trappe, 1992). However, for the purpose of this review the definition of endophyte will include 'fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease' (Wilson, 1995). Recently, it has been reported that endophytic bacteria may promote plant growth and suppress the plant diseases probably by means similar to plant growth-promoting rhizobacteria (PGPR).

Therefore, a better understanding of endophytic bacteria may help to elucidate their functions and potential role more effectively in developing sustainable systems of crop production. The search for natural association and endophytic interaction of diazotrophs with grass species is considered very promising, especially in grasses that grow naturally with adverse environmental conditions. Evidence of significant biological nitrogen (N_2) fixation in economically important graminaceous species, particularly sugar cane (*Saccharum* sp.), rice (*Oryza sativa*) and forage grasses, such as kallar

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grass (*Leptochloa fusca*) has generated tremendous interest in endophytic N_2 fixation by non-legumes and over the last few years research in the area of biological nitrogen fixation (BNF) associated with cereals and grasses. These plants are considered excellent hosts to identify superior endophytes that may potentially impact the crop growth (Reinhold-Hurek and Hurek, 1998). The search for diverse plant growth-promoting (PGP) diazotrophic bacteria is gaining momentum as efforts are made to exploit them as bioinoculants for various economically important crops. Since the present study was undertaken to study the endophytes and their plant growth promoting characters from naturally occurring grasses of southern India.

MATERIALS AND METHODS

Isolation of endophytic diazotrophs

Based on their predominance in each physiographic region, a total of 10 different weedy grass species were sampled (Table 1). Roots containing rhizospheric soil were washed with sterile distilled water, disinfected with 70% ethanol, rinsed, disinfected superficially with 3% sodium hypochlorite, rinsed again to eliminate hypochlorite, and spread on nutrient agar to confirm root surface sterility at 30°C for 5 days. Finally, roots were added with 0.9% NaCl (1:10) and macerated with mortar and pestle. One gram of macerated tissue was placed in a tube containing 9 ml sterile 0.9% NaCl. Specimen of washed roots, stems and leaves were macerated and 0.1 ml of serial dilutions in 4% (commercial) sugar solution up to 10^{-6} and there dilutions were inoculated into vials containing selective N-free semi solid media *viz*., NFb **(**Döbereiner, 1989), JNFb (Kirchhof *et al*., 1997), Rennie (Rennie, 1980), LGI (Reis *et al*., 2000) . After four to six days of incubation at 30°C, the population size was estimated by the MPN method and pellicle forming bacteria were subjected to further purification by streaking on N free agar plates. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at –80°C.

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 hexadecyl-trimethyl ammonium bromide (CTAB) method (Melody, 1997) and dissolved in distilled water to a final concentration of 20 ng/ll and stored at 4ºC. 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 lM 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. The conditions of the polymerase chain reaction (PCR) were: 0.5 min at 94ºC, 1 min at 50ºC, and 0.5 min at 72ºC with 40 cycles. The amplified products were resolved on a 1.5% agarose gel in 19 TBE buffer and documented in InGenius (Syngene, UK) documentation and analysis system.

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) 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).

Determination of Plant Growth-Promoting Traits

To quantify the IAA, One ml of the cultures at exponential stage was inoculated in 100 ml LB medium containing filter sterilized L-tryptophan (0.01per cent 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 Gordon 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 Penrose and Glick (2003) and compared with a standard curve of ±-ketobutyrate (Sigma-Aldrich, U.S.A.). Production of siderophores by the bacterial isolates were performed on Chrome azurol S (CAS) agar plates and incubated for 24h at room temperature (Schwyn and Neilands, 1987). The formation of bright zone with yellowish fluorescent colour 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). Solubilization of insoluble phosphates (Katznelson and Bose, 1959), potassium and zinc (Bunt and Rovira, 1955) were assayed. Bacteria having solubilization potential were identified by the appearance of a clear halo around colonies against an opaque background.

Gnotobiotic experiment

To study the impact of diazotrophic isolates on the growth of rice plants, dehulled seeds of rice (cultivar ADT 43) were surface sterilized by immersion in 70 % ethanol for 30 seconds, followed by soaking in 0.2 % mercuric chloride for 30 seconds and then washed with 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 strains 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 27ºC (HECO plant growth chamber). Shoot length, root length and dry biomass was recorded at 21 days after sowing.

Observation of endophytic bacterial colonization of rice seedlings by scanning electron microscopy (SEM)

The roots and culms from 15 days old fresh rice seedlings were cut, fixed with 3 % (v/v) glutaraldehyde, for 2 h at 4°C and washed with 0.1M phosphate buffer (pH 7.2) at room temperature for 10 min (three times). The samples again post fixed in 1% (w/v) osmium tetroxide in the same buffer for 2 h at 4°C. The fixed samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 75% and 100%) for 5 minutes in each concentration. Then the samples were treated with CO_2 and mounted on an aluminum cylinder with silver paste, and finally covered with a steam of carbon and ionized gold (Nowell and Parules, 1980). The samples were examined under a SEM (ICON, anaytical FEI Quanta 200, USA) operated at 15 kV at an 8-10 mm distance. Colonizing ability of the endophytic isolates in rice seedlings were documented as microphotographs.

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). Statistically significant differences between the treatments were analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at 5 % significance level.

RESULTS AND DISCUSSION

Plant-associated bacteria play key roles in their hosts' adaptation to changing environments in various ecosystems. These interactions between plants and beneficial bacteria can significantly affect general plant health and soil quality. Associative nitrogen (N_2) fixing bacteria may benefit their hosts by acting as N_2 biofertilizers and plant growth promoters. Several endophytic

Table 1.Details of grass species used in the present study

Grass species	Sampling site	Latitude	Longitude	Physiographic region
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
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
Setaria verticillata (Bristly foxtail)	Thirupoondi, Nagapattinam, Tamil Nadu	79° 53´ 37.6" E	10° 46´ 25.67" N	Coastal plains

Table 2. Isolation media, molecular characteristics and nitrogen fixing potential of isolates

Isolate code	Grass species	Isolation media[a]	Species homology	Percent homology	GenBank Accession No	Nitrogenase activity[b]	*nifH* gene
PRE2	*P. repens*	LGI	*Pantoea* sp.	98	KF906837	90.33 (± 6.51)[e]	+
CRE9	*C. rotundus*	Rennie	*Serratia* sp.	99	KF906838	88.38 (± 4.14)[e]	+
CRE10	*C. rotundus*	Nfb	*Serratia* sp.	96	KF906839	157.32 (± 15.41)[b]	+
CBE9	*C. barbata*	Nfb	*Bacillus* sp.	99	KF906840	166.25 (± 21.56)[a]	+
ORE3	*O. rufipogon*	Rennie	*Klebsiella pnemoniae*	94	KF906842	126.89 (± 23.24)[d]	+
ORE7	*O. rufipogon*	Rennie	*Enterobacter* sp.	97	KF906841	116.17 (± 20.8)[c]	+
SVE9	*S.vetricillata*	Rennie	*Enterobacter sacchari*	99	KF906843	83.37 (± 4.67)[f]	+

[a] Description was given in the materials and methods section;[b] nmoles ethylene/h/mg protein/h; 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 ($p \leq 0.05$)

Table 3. Plant growth promoting characteristics of endophytic isolates

Isolate	IAA[a]	ACC deaminse[b]	Siderophore (Catechol type)[a]	HCN[a]	Antogonism
Pantoea sp. (PRE2)	26.3 (± 0.27)[c]	95.8 (± 8.56)[c]	16.8 (± 1.16)[d]	55.2 (± 5.25)[b]	*R. solani*, *S. oryzae*, *P. oryzae*
Serratia sp. (CRE9)	19.6 (± 1.54)[e]	ND	19.2 (± 1.25)[c]	43.8 (± 2.54)[c]	*R. solani*, *S. oryzae*
Serratia sp.(CRE10)	27.7 (± 1.52)[h]	67.6 (± 3.18)[d]	35.5 (± 1.22)[a]	68.4 (± 6.58)[a]	*R. solani*, *S. oryzae*, *P. oryzae*
Bacillus sp.(CBE9)	31.6 (± 2.16)[b]	133.5 (± 8.19)[a]	28.5 (± 1.30)[b]	33.8 (± 2.54)[d]	*R. solani*, *S. oryzae*, *P. oryzae*
Enterobacter sp.(ORE7)	37.8 (± 1.24)[a]	115.8 (± 9.53)[b]	17.5 (± 1.32)[d]	54.9 (± 5.18)[b]	*R. solani*, *S. oryzae*, *P. oryzae*
K. pnemoniae (ORE3)	23.4 (± 0.19)[d]	ND	ND	ND	*S. oryzae*
E. sacchari (SVE9)	31.8 (± 1.24)[f]	63.5 (± 4.04)[d]	16.4 (± 1.24)[d]	43.4 (± 1.19)[c]	*S. oryzae*

Values are mean (± SE) (n=3); ND- not detected; [a]mg mg^{-1} protein; [b]nmoles of a-ketobutyrate mg^{-1} protein h^{-1}

bacteria enhance growth and improve general plant health (Sharma and Nowak, 1998). Many plant-growth promoting bacteria (PGPB), including a diverse group of soil bacteria, are thought to stimulate plant growth by various mechanisms such as protecting plants against pathogens, providing plants with fixed N_2 (Iniguez *et al*., 2004), producing plant hormones, or enhancing mineral availability in the soil (Sessitsch *et al*., 2002).

The endophytic isolations are performed in nitrogen-free semisolid media with different carbon sources and pH values. Altogether thirty two endophytic diazotrophic isolates were obtained by using four N-free media after 5 days of incubation. Among the different diazotrophic growth media tested, Rennie medium are able to produce the maximum number of isolates. This semisolid nitrogen free media offer the possibility for diazotrophic bacteria to find the right niche for nitrogen fixation. The inclusion of N-free modified Rennie medium for isolation of diazotrophs turned out to be very useful, as the endorhizosphere bacteria could only be detected on this medium, indicating that these bacteria may be adapted to high salt concentrations. The present finding are in agreement with Barraquio *et al*. (1997) and Elbeltagy *et al.* (2001) reports on the isolation of nitrogen fixing bacteria from N-free media.

These 32 isolates were used for further re-infection study (Koch's postulates). Only seven bacterial isolates were re-isolated from gnotobiotically grown rice seedlings. Unique bacterial colonies from different grass species were further reconfirmed as putative diazotrophs by nitrogenase activity and PCR. Based on such data, seven isolates were selected for further study.

All the authenticated seven diazotrophic endophytes were further phylogenetically identified using 16S rRNA gene sequence homology revealed the presence of diversity of Gamma proteobacteria and Firmicutes (Table 2). The nitrogenase ranged from 83.37 to 166.25 n moles ethylene/mg protein/ h. and the highest nitrogenase activity was exhibited by isolate *Bacillus* sp.CBE9 (166.25 ±21.56 n moles 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

Table 4. Mineral solubilizing potential of endophytic diazotrophs

Isolate	Phosphorus		Potassium		Zinc	
	Solubilization efficiency (%)	Available P (µg/ml)	Solubilization efficiency (%)	Available K (µg/ml)	Solubilization efficiency (%)	Available Zn (µg/ml)
Pantoea sp. (PRE2)	233 (±12.26)^{a}	0.78 (± 0.01)^{a}	100 (± 11.16)^{b}	2.8 (± 0.04)^{a}	133 (±12.10)^{b}	0.60 (± 0.04)^{a}
Serratia sp. (CRE9)	250 (±12.58)^{a}	0.56 (± 0.03)^{c}	ND	ND	ND	ND
Serratia sp.(CRE10)	75 (± 21.45)^{c}	0.36 (± 0.04)^{de}	100 (± 22.10)^{b}	2.7 (± 0.04)^{a}	100 (± 12.16)^{c}	0.54 (± 0.04)^{b}
Bacillus sp.(CBE9)	167 (±12.94)^{b}	0.40(±0.02)^{d}	120 (± 21.15)^{b}	2.9 (± 0.08)^{a}	100 (± 25.12)^{c}	0.59 (± 0.24)^{b}
K.pnemoniae (ORE3)	167 (±12.94)^{b}	0.65(± 0.05)^{bc}	ND	ND	ND	ND
Enterobacter sp.(ORE7)	200 (±12.10)^{ab}	0.56 (±0.04)^{c}	133 (± 12.14)^{a}	2.3 (± 0.03)^{b}	150 (± 2.14)^{a}	0.22 (± 0.01)^{c}
E. sacchari (SVE9)	100 (± 21.17)^{c}	0.23 (± 0.01)^{e}	ND	ND	ND	ND

ND- solubilization not detected. 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 ($p \leq 0.05$);

confirmed that all were able to amplify the partial *nifH* gene. In the present investigation, *Enterobacter* sp. accounts for 86% of the total diazotrophs members of enterobateriales are known N_2-fixers and one of the most universal of endophytic genera particularly in grasses. *Enterobacter* has been identified as endophytes of several plants such as *Citrus sinensis*, soybean, sweet potato and maize (Araújo *et al*., 2002; Zinniel *et al*., 2002 and Kuklinsky-Sobral *et al*., 2004). Doty *et al*. (2009) isolated diazotrophic endophytes *Pantoea* sp. and *Enterobacter* sp. from grasses. In wheat, Iniquez *et al*. (2004) demonstrated and confirmed the nitrogen fixing activity of *K. pneumoniae*. The nitrogen fixing activity of *K. pneumoniae* isolates are again confirmed by our work.

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 PGPR activity along with diazotrophy is agreement with our results. After establishing in a plant, endophytes can positively influence plant growth and its resistance to different stresses. It is likely that some diazotrophic bacteria stimulate plant growth both by supplying N and by production of phytohormones, in particular IAA. Members of the Enterobacteriaceae isolated from selected grass species were competent plant growth promoting traits, with the ability to fix nitrogen, produce IAA, GA and mineralize insoluble plant nutrients. They have earlier been also shown to be potent biological control agents against fungal diseases.

Apart from nitrogen fixation, the diazotrophic endophytes of all genera used in the present study possess different traits related to plant growth promoting, which are described in Table 3. All the diazotrophic isolates gave positive result with regard to IAA production and the maximum amount of IAA was produced by *Enterobacter* sp. ORE7 (37.8 ±1.24 µg/mg protein). The organisms exhibited both diazotrophic character and producer of IAA which make them good candidates for crop growth. 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 (Vasquez *et al*.,

Table 5. Abiotic stress tolerance by the endophytic diazotrophic isolates

Isolate	Abiotic stress		
	NaCl (%)	Temperature°C	Heavy metal (µg ml^{-1})
Pantoea sp. (PRE2)	5	50	$Cd^{2+}_{(100)}$, $Co^{2+}_{(200)}$
Serratia sp. (CRE9)	7.5	55	$Co^{2+}_{(100)}$
Serratia sp.*(*CRE10)	5	50	$Cd^{2+}_{(100)}$, $Zn^{2+}_{(200)}$, $Co^{2+}_{(200)}$
Bacillus sp.(CBE9)	7.5	55	$Hg^{2+}_{(100)}$, $Zn^{2+}_{(200)}$, $Co^{2+}_{(200)}$
K. pnemoniae (ORE3)	5	50	$Cd^{2+}_{(100)}$
Enterobacter sp.(ORE7)	5	50	$Hg^{2+}_{(100)}$, $Zn^{2+}_{(100)}$, $Co^{2+}_{(200)}$, $Ni^{2+}_{(200)}$
E. sacchari (SVE9)	7.5	55	$Cd^{2+}_{(100)}$, $Hg^{2+}_{(100)}$, $Zn^{2+}_{(400)}$

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

Isolate	Shoot length (cm)	Root length (cm)	Plant dry matter (mg)
Pantoea sp. (PRE2)	13.2 (± 1.23)^{d}	6.7 (± 0.55)^{c}	41.4 (± 0.12)^{c}
Serratia sp.*(*CRE10)	14.8 (± 1.39)^{c}	6.5 (± 0.50)^{c}	41.3 (± 0.22)^{c}
Bacillus sp.(CBE9)	15.6 (± 1.49)^{b}	8.2 (± 0.65)^{a}	34.0 (± 0.23)^{b}
Enterobacter sp.(ORE7)	19.8 (± 1.22)^{a}	7.9 (± 0.71)^{b}	50.0 (± 0.12)^{a}
A. lipoferum Az 204*	12.8 (± 0.85)^{e}	6.8 (± 0.42)^{c}	30.0 (± 0.23)^{d}
Control	12.5 (± 1.22)^{e}	5.8 (± 0.59)^{d}	23.0 (± 0.21)^{e}

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 ($p \leq 0.05$), *standard culture

2000 and Canbolat *et al*., 2006) and biocontrol properties (Kloepper *et al*., 2004 and McSpadden-Gardener, 2004) by different strains of *Bacillus* have also been widely reported and were again confirmed by our work. In nature, 1-aminocyclopropane-1-carboxylate 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, five isolates were able to produce ACC deaminase, where the isolate *Bacillus* sp. CBE9 registered the highest activity of 133.5 ± 8.19 n moles a-ketobutyrate /mg protein/h. With regard siderophore and hydrogen cyanide production the isolate *Serratia* sp. CRE10 recorded the

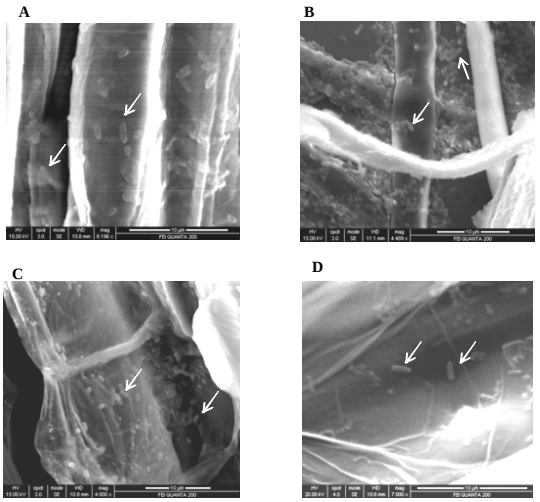

Fig. 1. Colonization of diazotrophic bacterial strain (*Serratia* sp. CRE2) on rice seedlings. A- Longitudinal section of culm; B- Surface of the root; C,D- Longitudinal section of root. Arrow heads show the presence of bacteria

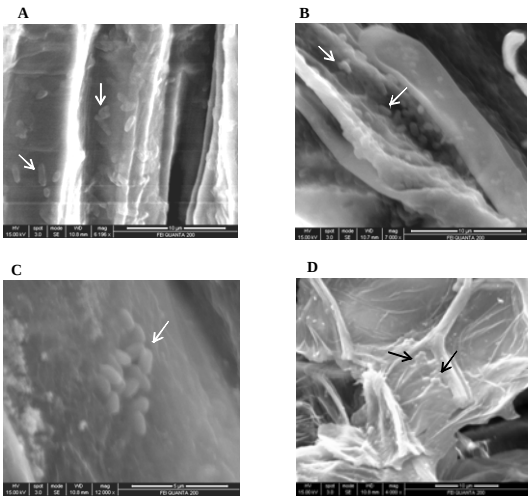

Fig 2. Colonization of diazotrophic bacterial strain (*P. agglomerans* - ORE9) on rice seedlings. A- longitudinal section of culm; B- surface of the root; C,D- longitudinal section of root. Arrow heads show the presence of bacteria

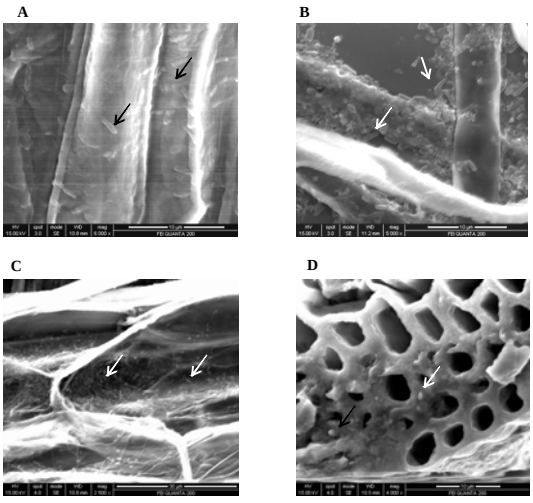

Fig. 3. Colonization of diazotrophic bacterial strain (*Pseudomonas* sp. CRE10) on rice seedlings. A-longitudinal section of culm; B- surface of the root; C-longitudinal section of root; D- transversal section of root. Arrow heads show the presence of bacteria

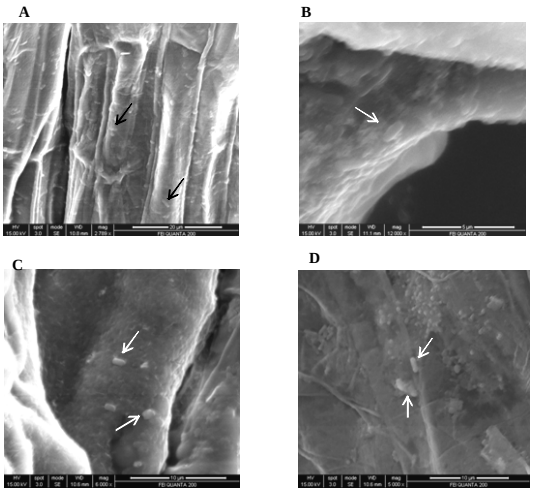

Fig 4. Colonization of diazotrophic bacterial strain (*Enterobacter* sp. ORE7) on rice seedlings. A-longitudinal section of culm; B- surface of the root; C,D- longitudinal section of root. Arrow heads show the presence of bacteria

maximum of 35.5 ± 1.22 and 68.4±6.58 µg/mg protein respectively.

Diazotrophic isolates exhibited significant growth-inhibitory activity against a range of phytopathogenic fungi such as *viz.*, *Rhizoctonia solani*, *Pyricularia oryzae* and *Sarocladium oryzae*. Preliminary screening of antagonistic activity of all the isolates confirmed that out of 7 isolates, 3 isolates (*Serratia* sp. CRE10, *Bacillus* sp. CBE9 and *Enterobacter* sp. ORE7) had antagonistic potential against all the three pathogens.

The results of the present study on mineral solubilization have revealed that diazotrophic isolates *Pantoea* sp. (PRE2), *Serratia* sp. CRE10, *Bacillus* sp. CBE9 and *Enterobacter* sp. ORE7 solubilize all three minerals effectively (Table 4). Similarly, a positive correlation between the potential for P,K and Zn solubilization has been reported (Wani *et al*. 2007).

The multifaceted plant growth promoting diazotrophs were evaluated for their growth under different stress conditions and the results are reported in Table 5. The isolates *Serratia* sp. CRE9, *Bacillus* sp. CBE9, and *E. Sacchari* SVE9 are tolerance to higher temperature and salt. The maximum tolerable concentration (MTC) of 5 different heavy metal ions *viz.*, Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} by diazotrophic isolates was estimated by streaking the cultures on to LB medium containing heavy metal ions. The isolates *Serratia* sp. CRE10, *Bacillus* sp. CBE9, *Enterobacter* sp. ORE7 and *E. Sacchari* (SVE9) were found to have higher resistance heavy metals. Jenson (1981) reported

N_2 fixing microorganisms survive during hot dry conditions upto 60ºC. In the present study, gnotobiotic assay inoculation effects of diazotrophic bacteria on rice were studied. It clearly revealed that diazotrophs can aid in production of healthy and vigorous seedlings rice. All of the strains selected for gnotobiotic study showed significant nitrogenase activity, and amplification of the *nifH* gene confirmed their ability to fix nitrogen. Production of maximum shoot length and plant dry matter was observed for the plant treated with *Enterobacter* sp. ORE7 (19.8 ± 1.22cm and 50.0 ±0.12mg). While, higher root length was recorded in *Bacillus* sp. CBE9 (8.2 ± 0.65 cm) (Table 6). Bioinoculants increase crop growth by a combination of mechanisms, which include biological nitrogen fixation (BNF), phytohormone production, increasing the availability of soil nutrients, and disease control (Cocking, 2003).

The endophytic colonization in the rice seedlings (roots and culm) were examined by SEM analysis. Results indicated that endophytic bacteria were preferentially colonized in the rhizoplane of rice roots (Fig 1-4). Diazotrophic cells were observed in the longitudinal and horizontal sectioned roots and culms. This finding is probably due to the fact that intercellular regions represent more space and opportunity for the movement of endophytes; besides, very probably the mucilaginous layer, which covers the epidermis of the root, has a lower tension in these regions in accordance with Bowen (1979). The close association between a plant and an endophyte may provide suitable conditions for nutrient transfer between the bacteria and their host, than the association between predominantly rhizosphere bacteria and plants (Stoltzfus and de Bruijn, 2000). Previous reports indicate that, at the intercellular regions, there is an important increase in the concentration of carbon as a source of energy, thus explaining the preference of bacteria for this part of the root. Islam *et al*. (2009) reported that inoculation of rice with free-living diazotrophic bacteria remarkably increased plant height and dry biomass production compared with the control under greenhouse conditions. George *et al*. (2013) found that significant increase in growth accompanied with higher populations of plant beneficial microorganisms in their rhizospheres were recorded on inoculation with endophytic diazotrophs.

CONCLUSIONS

The present investigation, unravel the diversity richness of diazotrophic bacteria colonizing in the internal tissues of naturally growing grass species in different parts of southern India. The present work suggest that exploring those elite diazotrophic strains having multiple plant growth promoting traits, as bioinoculants for nutrient management and for biotic and abiotic stress mitigation and sustainable crop production with fewer chemical inputs.

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