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Conventional and molecular identification of bacterial endophyte: *Gluconacetobacter diazotrophicus* from different Sugarcane cultivars

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Abstract
The bacterial endophytes are plant-associated and they can survive in the internal tissues of the plant without harming the host plant. The endophytes play a vital role in plant growth promotion as they directly or indirectly promote plant growth. The genus *Gluconacetobacter* encompasses different species which are immensely used in agricultural and industrial sectors. This study aims to identify the presence of *Gluconacetobacter diazotrophicus* and in sugarcane crop considering their potential biotechnological applications. Eighteen isolates of *Gluconacetobacter diazotrophicus* were obtained from the root, bud, and leaf portions of sugarcane varieties (CoC 92016, Co 86032, Co 86032, Co 8608 and Co 86034) collected from Thondamuthur, Sathyamangalam, Aaapakodal of Coimbatore and Erode districts, Sugarcane Breeding Institute, Coimbatore, and Eastern Block of Tamil Nadu Agricultural University, Coimbatore. The isolates are identified biochemical evaluations, and molecular and phylogenetic analyses. These isolates were characterized and confirmed by comparing with the standard type strains PAL 5 and L5. All the isolates produced orange yellow colonies on LGI and Acetic LGI agar plates and chocolate brown colonies on Potato agar plates. Based on the colony morphology and biochemical analysis best four isolates are selected for the 16S r RNA analysis. The phylogenetic characterization of 16S r RNA gene sequencing analysis of isolates showes very distinct to other closely related genera of *Gluconacetobacter diazotrophicus.* The isolates have ecological significance and influence the advance research towards the evaluation of plant-soil interactions in sugarcane eco systems.

Keywords: *Gluconacetobacter diazotrophicus*, endophytes, sugarcane, phylogenetic analysis

1. Introduction
During the recent year, the increasing interest to promote eco-friendly safe fertilizers to the farming community is a task based assignment. Apparently, researchers concentrate on efficient bio based fertilizers mainly plant growth promoting bacteria (PGPB) [1]. The family PGPB which includes free living bacteria, which enormously available in any eco system. The plant tissue associated PGPB referred as ephiphytes, and the endophytes, which colonize the interior of plant tissues without causing apparent damage to the host. Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria or beneficial plant growth promoting rhizobacteria (PGPR) as a result of direct and indirect effects [2]. There are several ways by which plant growth promoting bacteria can benefit the plant directly, e.g. by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones). Indirectly, plant growth promotion occurs due to the improved soil conditions and reduced incidence of pests and diseases.

2. Materials and Methods
2.1 Reference culture collection
Gluconacetobacter diazotrophicus type strain PAL 5 obtained from Dobereiner Lab, University of Embrapa Brazil along with *G. diazotrophicus* native isolate, L5 cultured at the Biofertilizer Production and Quality Control Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore were used in the study.

2.2 Isolation of *G. diazotrophicus*
G. diazotrophicus cultures were isolated from the sugarcane samples using the standard protocol [3]. One gram of the sugarcane samples (root / leaf / stem / bud) were washed thoroughly in running tap water, placed in 70 per cent alcohol for 15 seconds and immediately washed in sterile distilled water for 3 to 4 times repeatedly.

The surface sterilized samples were macerated in a sterile pestle and mortar along with few acid washed sand particles. A drop of the suspension as such and 10^{-2} dilution of it was inoculated into various enrichment media *viz*., semisolid diluted cane juice medium, semisolid LGI medium and semisolid acetic LGI medium supplemented with yeast extract (20 mg l^{-1}) The tubes were incubated at room temperature without disturbance until the formation of subsurface pellicles.

2.3 Characterization of *G. diazotrophicus*

The isolated cultures were grown in acetic LGI medium and single colony was streaked on acetic LGI agar slants and the young cultures at exponential phase i.e. on 7th day were taken for further characterization.

2.3.1 Gram staining

Gram staining was carried out as per Huker's modified method [4].

2.3.2 Motility

The motility of the isolated cultures was observed by hanging drop technique using a cavity slide [5].

2.3.3 Oxidase test

Small pieces of filter paper were soaked in one per cent aqueous tetra methyl-p-phenylene diamine and placed in a petri dish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after 10 seconds indicated positive oxidase test whereas development of a light violet colour indicated negative oxidase test.

2.3.4 Nitrate Reductase test

Cultures were inoculated in test tubes containing nutrient glucose broth with one per cent KNO_3 and incubated at 37° C for 48 h. Test for the presence of nitrate reductase was carried out by adding one drop of sulfanilic acid and 1 drop alpha naphthylamine reagent to each of the nutrient broth cultures. Development of distinct red colour indicated positive test and no colour development indicated negative test.

2.3.5 Test for hydrogen sulphide formation

Peptone iron broths in tubes were inoculated with cultures and incubated at 37°C for 48 h. Black precipitation in the medium indicated hydrogen sulphide formation.

2.3.6 Catalase test

Loopful of bacteria was taken from the solid medium and mixed with a drop of 3 per cent hydrogen peroxide on a glass slide. Catalase positive organisms exhibited bubbles of oxygen formation.

2.4 Identification of siderophore producing *Gluconacetobacter* species by 16S r RNA sequencing

2.4.1 Genomic DNA extraction

The genomic DNA from the eighteen isolates was isolated using the standard protocol of hexadecy-trimethyl ammonium bromide (CTAB) method[6]. About 25 ml of quantity actively grown culture was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4°C. The supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells (to remove extracellular materials). Centrifuged at 5000 rpm for 5 min at 40C, the supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of butanol. The pellet was again resuspended in 1 ml TE, buffer added with 100 µl lysozyme (10 mg ml-1 freshly prepared) and incubated at room temp for 5 min. After incubation, 100 µl of 10 per cent SDS and 25 µL of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for 1 h. To this 200 µl 5 M of NaCl was added and homogenized. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65 ^{0}C for 10 min. The mixture was extracted with one ml of phenol: chloroform mixture, mixed well and centrifuged at 6000 rpm for 15 min at 4 ^{0}C. The aqueous layer was transferred carefully to a 2.0 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated one h to overnight at – 20 °C. The DNA was pelletized by centrifugation at 12000 rpm for 15 min at 4 ^{0}C. The pellet was washed with 70 per cent ethanol, dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One µl DNAse free RNAse (10 mg per ml) was also added by swirling and incubated at 37^{0}C for 30 min. The DNA was stored at – 20 ^{0}C for further use.

2.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the quality of DNA and also to separate the products amplified through polymerase chain reaction [7]. About 1X TAE tank buffer in 500 ml quantity was prepared to fill the electrophoresis tank and to prepare the gel. In a separate conical flask, agarose (0.8 per cent for genomic DNA and 1.5 per cent for PCR product) was added to 1X TAE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature. Ethidium bromide was added at the rate of 5 µl 100 ml^{-1} to agarose solution and was allowed to mix completely. It was poured into the gel mould and the comb was placed properly allowed to solidify for half an hour at room temperature. After solidification the comb was removed carefully. The caste gel was placed in the electrophoresis tank containing 1X TAE buffer with the well near the cathode and submerged to a depth of 1 cm. Fifteen µl of the PCR product was mixed with 3µl of 6X tracking dye and mixed well by pipetting in and out for 3 times. The mixture was loaded into the wells with the help of the micropipette. Two µl of 1 kb DNA ladder (Fermentas, USA) was loaded in one of the wells as a standard marker.

2.4.3 Amplification of DNA

The presence of 16S r RNA gene in the isolates was detected by partial amplification of the gene using specific primers. 30 µl PCR reaction mixture contain DNA template 50 ng, 1XTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 1.5mM $MgCl_2$ and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) using conditions: initial denaturation at 95° C for 1 min, 35 cycles consisting of 94°C for one min (denaturation), 60°C for one min (annealing), 72°C for one min (primer extension) and final extension 72°C for 5 min.

2.4.4 Sequencing analysis

The 16S r RNA genes was amplified and the band of the expected size was gel-purified using spin columns (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 in an automated sequencer Applied Biosystems (Model 3100) Ireland. The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers EF116582 to EF116592.

2.4.5 Phylogentic analysis

The identity of 16S r RNA gene sequence was established by performing a similarity search against the Gene Bank database [8]. The phylogentic tree was constructed with existing *G.diazotrophicus* gene sequence from different proteobacteria which was obtained from NCBI. The phylogenetic tree was constructed by neighbor-joining method [9]. Of using MEGA 4.0 and the tree file was analyzed using tree view [10].

3. Result

The *G. diazotrophicus* was isolated from sugarcane varieties (CoC 92016, Co 86032, Co 86032, Co 8208, Co 86034) covering major sugarcane tracts of Tamil Nadu. Eighteen isolates were detected from different parts of sugarcane *viz*., stem, bud and root (Table. 1) using standard morphological characteristics of the *G. diazotrophicus*.

Table 1: *Gluconacetobacter diazotrophicus* isolates from different sugarcane varieties

Location	Variety	Plant part	Isolate [a]
Thondamuthur Coimbatore district, India	COC 92061	Root Bud	GD 1, GD 2, GD 3, GD 4
Sugarcane Breeding Institute, Coimbatore, India	Co 86032	Leaf Root	GD 5, GD 6, GD 7, GD 8
Sathyamangalam Erode district, India	Co 86032	Leaf Root	GD 9, GD 10, GD 11, GD 12
Tamil Nadu Agricultural University, Coimbatore Eastern block, India	Co 8208	Root Leaf Bud	GD 13, GD 14, GD 15, GD 16, GD 17
Aaapakoodal, Erode district, India	Co 86034	Root	GD 18
Standard strain 1			PAL 5
Standard strain 2			L 5

[a] *G.diazotrophicus* were isolated using N-free LGI medium and authenticated by PDA medium

The visual observations indicated that all the eighteen isolates had shown to exhibit typical heavy orange yellow surface pellicles on colourless LGI and acetic LGI semisolid mediums and chocolate brown colour in PDA medium. The isolated *G. diazotrophicus* strains were confirmed by performing characterization tests *viz*., gram reaction, motility, catalase activity, oxidase activity, nitrate reductase activity, hydrogen sulphide formation and growth under different conditions (Fig 1& Table 2).

Fig 1: Colony morphology of G. *diazotrophicus* isolates in LGIP and PDA medium

Table 2: Biochemical Characterization of *G. diazotrophicus* isolates

Gd. Isolates	Gram reaction	Catalase Activity	Oxidase Activity	Nitrate reductase activity	H_2S formation
GD 1	G-*ve*	+	+	-	+
GD 2	G-*ve*	+	+	-	+
GD 3	G-*ve*	+	+	-	+
GD 4	G-*ve*	+	+	-	+
GD 5	G-*ve*	+	+	-	+
GD 6	G-*ve*	+	+	-	+
GD 7	G-*ve*	+	+	-	+
GD 8	G-*ve*	+	+	-	+
GD 9	G-*ve*	+	+	-	+
GD 10	G-*ve*	+	+	-	+
GD 11	G-*ve*	+	+	-	+
GD 12	G-*ve*	+	+	-	+
GD 13	G-*ve*	+	+	-	+
GD 14	G-*ve*	+	+	-	+
GD 15	G-*ve*	+	+	-	+
GD 16	G-ve	+	+	-	+
GD 17	*G-ve*	+	+	-	+
GD 18	*G-ve*	+	+	-	+
PAL 5	*G-ve*	+	+	-	+
L 5	*G-ve*	+	+	-	+

The metabolic activities of the isolated were used as a hue to characterize the *G. diazoteophicus* isolates. In this regard, nitrate reductase, catalase, oxidase and the production H_2S were used to knock out correct isolates. Among the characterizations testes undertaken, catalase, oxidase and the production H_2S were found to show positive results suggesting the presence of *G. diazotrophicus*.

Authentication of *G. diazotrophicus* isolates by amplification of partial 16S r RNA gene. After the characterization was performed in all the eighteen isolates, these were cultured in

LB broth and found that three strains namely GD 1, GD 5 and GD 8 exhibited luxuriant growth. These strains were amplified in the presence of 16S r RNA gene and detected using specific primers. The total genomic DNA extracted from the *G. diazotrophicus* isolates (GD 1, GD 3 and GD 5) was resolved in agarose gel electrophoresis and presented in (Fig. 2). This suggested that the DNA was intact and uniform in all three isolates and were amplified using PCR. The PCR amplification of partial 16S r RNA gene of the isolates is presented in (Fig 3).

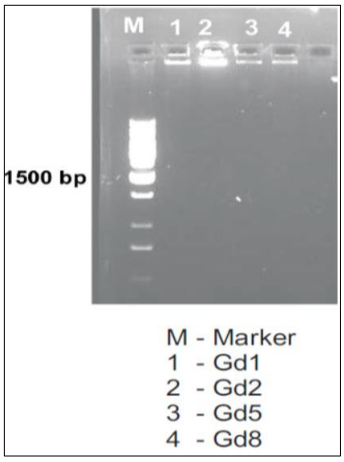

Fig 2: Genomic DNA isolation of *G. diazotrophicus* isolates

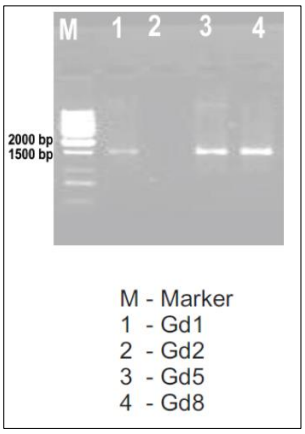

Fig 3: 16s rDNA amplification profile of *G. diazotrophicus* isolates

Phylogentic characterization of 16S r RNA gene from *G.diazotrophicus* isolates. The partial 16S r RNA amplified from *G. diazotrophicus* GD 1, GD 3 and GD 5 isolates was sequenced and phylogenetic tree was constructed with existing 16S r RNA gene from different proteobacteria the bacterial species, strain name and the accession number of the gene are presented in the phylogenetic tree. The data showed that 16S r RNA gene was very distinct to *G. diazotrophicus*. These isolates form a very distinct clustering in phylogenetic tree of 16S r RNA by Neighbor-Joining method as presented in (Fig. 4).

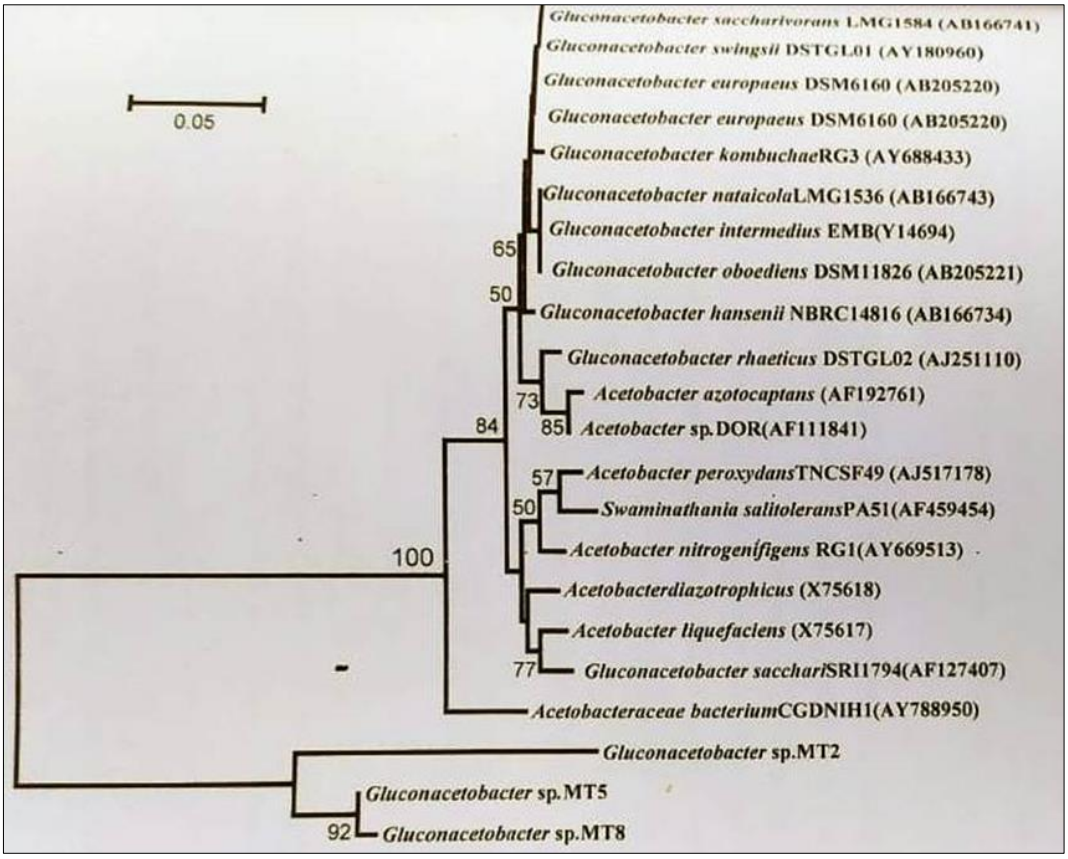

Fig 4: Neighbour-joining tree based on analysis of partial 16S rRNA nucleotide sequences of *G.diazotrophicus*. Phylogenetic tree showing the relatedness of *Gluconacetobacter* species isolated from gene bank are compared with existing *Gluconacetobacter* related species. V Bootstrapping values of 500 or more are indicated as present at the nodes.

4. Discussion

The isolates developed yellow surface pellicles on LGI and acetic LGI semisolid medium indicating the microaerophilic nature of this organism [3]. The yellow colour of the isolate may be attributed to assimilation of bromothymol blue in the medium and strong acid production and indicating acidic

condition to grow [11, 12]. All eighteen isolates screened in the study were found to produce yellow to dark yellowish orange surface pellicles on semisolid LGI and acetic LGI medium and brown pigmented colonies on potato agar medium. The over-oxidation of sucrose in the PDA media caused brown coloration while yellowish coloration in LGIP. Whereas, the hydrogen sulphide evolution by *G. diazoteophicus* as a consequence of cystein production at acidic condition. The isolates were gram negative and rod shaped, motile, and catalase and oxidase positive exhibiting reactions. The nitrate reductase activity in the isolates appeared to show negative results which may be due to the non-utilization of nitrate from the added source. Since the nitrate reducase is a substrate inducible enzyme it is quite obvious that the hardly utilizable form of nitrate would have resulted in negative [12].

It has been shown that the *G. diazoteophicus* can amplify about 550 bp size partial 16S r RNA gene. In order to assess the evolutionary relationship among the isolates, a phylogenetic tree was constructed using the 16S rRNA sequencing data. The data clearly demonstrated that siderophore producing *Gluconacetobacter* isolates from sugarcane were distinct in comparison to *G. diazotrophicus* and all three isolates such as GD 1, GD 3 and GD 5 were close to each other. The DNA-DNA reassociation values for siderophore producing *Gluconacetobacter* isolates were 60 per cent in comparison to the existing phylogenetic tree of *G.diazotrophicus*. The data are in agreement with the findings of [13] reported that siderophore producing *Gluconacetobacter* isolates species were unique to the *Gluconacetobacter*. The DNA-DNA- re-association was 70 per cent which supposedly 97 per cent as threshold for 16S r RNA similarity level for the delineation of bacterial species [14]. On the other hand, the DNA-DNA re-association level between *G. swingssii* and *G. rhaeticus* isolated form Italian apple fruit was less than 60 per cent which was very distinct to other *G.diazotrophicus* isolates [15].

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