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Isolation and screening of nitrogen fixing endophytic bacterium *Gluconacetobacter diazotrophicus* GdS25

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A B S T R A C T

The present study is on the isolation, characterization and screening of endophytic bacterium i.e., *Gluconacetobacter diazotrophicus* which was isolated from the tissues of surface sterilized roots of four different crops viz., sugarcane, maize, pineapple and carrot. The morphological and biochemical tests permitted characterization of *Gluconacetobacter diazotrophicus* isolates. Further the plant growth promoting traits such as nitrogen fixing ability, production of phytohormones (Indole acetic acid and Gibberellic acid), phosphorus and zinc solubilization and antagonistic activity by dual culture method were assessed. Out of 113 isolated screened only 10 efficient isolates from various crops were selected based on nitrogen fixing capacity. An isolate from sugarcane GdS25 fixed maximum nitrogen *i.e.*, 147µg of N/mg of Carbon used and produced maximum concentration of Indole acetic acid (4.94µg/ml) and Gibberellic acid (7.1µg/25ml) respectively. GdS25 showed phosphorus solubilization zone of hydrolosis 2.1cm where as zone of zinc solubilisation of 3.5cm. An isolate from sugarcane GdS25 exhibited 83% inhibition against *Rhizoctonia bataticola* and 88% against *Exerohilium maydis*. The GdS25 is more potent as compared to the reference strain *G. diazotrophicus* MTCC1224.Five efficient isolates *viz.*, GdS25, GdS26, GdM5, GdM6 and GdP7 will be taken up for further studies.

Introduction

Gluconacetobacter diazotrophicus is an endophytic bacterium first isolated from the sugarcane growing regions of Brazil (Cavalcante and Dobereiner, 1988). It was widely studied and used as a model system to assess the bacterial endophyte – plant interactions. After its first discovery, it was reported from variety of crops *viz.*, coffee (Jimenez-Salgado *et al*., 1997), ragi (Loganathan *et al.*, 1999), pineapple (Hernandez *et al.*, 2000) and a latest report states *Gluconacetobacter sp.* as a natural colonizer of the wild rice (*Porteresia coarctata* Tateoka, formerly *Oryza coarctata* Roxb.) and a salt tolerant Pokali rice variety (Loganathan and Nair, 2003). These reports clearly indicated the wide occurrence of *G. diazotrophicus* in different plants than initially expected.

The endophytic nitrogen fixation concept has been recently gaining momentum. The biologically fixed nitrogen can supplement the nitrogen requirement of the crops in case of N deficit soils. Another important trait beneficial to the plant health is the production

of growth hormones. *G. diazotrophicus* has the ability to produce both auxins and gibberellins in significant quantities (Bastian *et al*., 1998).

Now-a-days, screening organism for the phosphorus solubilizing ability is also considered as an agronomically important trait for an endophytic bacterial isolate (Suman *et al.,* 2001; Verma *et al*., 2001).

Apart from phosphorus, micronutrients like Zn, Fe and Mn were found to be deficient in most of the soils with Zn as a foremost nutrient throughout the world (Alloway, 2001). The soluble form of Zn applied to the soil gets transformed into different unavailable forms due to soil reaction.

Thus if an isolate has the ability to solubilize this insoluble form to soluble one, it will contribute significantly to the crop productivity. *G. diazotrophicus* exhibits antagonistic property against many bacterial and fungal pathogens. It has also been established that *G. diazotrophicus* exhibits antagonistic potential against *Colletrotrichum falcatum*, a causal organism of redrot in sugarcane (Mutthukumarsamy *et al*., 2002).

The recent findings state that properties other than nitrogen fixation like production of growth promoting substances, increased nutrient uptake, synthesis of plant growth modulation enzymes like ACC deaminase, enhanced stress resistance are the key factors for plant growth promotion of a diazotroph in the rhizosphere region (Dobbelaere *et al*., 2003). So a study was carried out with the aim to isolate *Gluconacetobacter diazotrophicus* from various crops, characterize them and further the growth promoting parameters like nitrogen fixing ability, growth hormone production, antagonistic activity, phosphorus and zinc solubilization were also assessed to screen the efficient strains to take them to pot culture experiment.

Materials and Methods

Locations of the sampling sites

The root samples of sugarcane, maize, pineapple and carrot were collected from Dharwad, Belgaum, Uttara Kannada and Shimoga districts of Karnataka, India. Dharwad district is situated in the western sector of the northern half of Karnataka which encompasses an area of 4263 km^2 lying between the latitudinal parallels of 15°02' and 15°51' North and longitudes of 73°43' and 75°35' East. The district is bounded on the north by the district of Belgaum, on the east by the district of Gadag, on the south Haveri and on the west by Uttara Kannada. Shimoga lies between the latitudes 13°27' and 14°39' N and between the longitudes 74°38' and 76°04' E at a mean altitude of 640 metres above sea level and spread over an area of 8465 km^2.

Media and cultural conditions

N-free semisolid LGI medium supplemented with 0.5% sugarcane juice at pH 4.5 was used (Cavalcante and Dobereiner, 1988). For isolation and culturing, acetic acid LGI agar plates supplemented with yeast extract (50 mg /1) and potato agar plates with 10% cane sugar were used (Cavalcante and Dobereiner, 1988).

Preparation of root samples

Since *G.diazotrophicus* is an endophyte, the isolation was done using root samples. The plants were uprooted and the root portion was separated and washed with tap water. The roots were washed with sterile distilled water and surface sterilized for 5 min with 5% sodium hypochlorite (NaOCl), and then washed five times with sterile distilled water.

Isolation of *G. diazotrophicus*

The surface sterilized root samples were weighed and homogenized in a sterile sucrose solution (1%) using a sterile pestle and mortar. Aliquots (500 ml) were inoculated in semisolid LGI (Cavalcante and Dobereiner, 1988) and incubated at 30°C for 4–6 days. Fifteen replicates from each plant part (root, stem, leaf, and rhizosphere) were inoculated in semisolid LGI tubes. Yellowish bacterial growth from the tubes was streaked onto LGI plates (Cavalcante and Dobereiner, 1988) and incubated at 30ºC for 6–7 days. The colony morphology was compared with reference strain *G. diazotrophicus* (MTCC culture 1224).

Biochemical characterization of isolates

The isolates presumably identified as *G. diazotrophicus* using LGI media were further characterized using a series of biochemical tests *viz*., Gram stain, motility, catalase, gelatin hydrolysis, over-oxidation of ethanol, brown pigment production on GYC agar, growth on carbon sources, growth at various concentrations of sugar and growth at various temperatures, according to Dong *et al.*, (1995) and Muthukumarasamy *et al*., (1999).

Evaluation of plant growth promoting activities

Nitrogen fixing ability: nitrogen estimation by microkjeldhal method

The 48 hour old cultures were inoculated to 5ml of N free semi solid broth of LGI medium. It was incubated for 48 hours. 1ml of this broth was inoculated to 50ml semisolid medium. Then it was incubated for 15 days. 10 ml of this culture was used for N estimation by following the standard procedure of Microkjeldhal technique (Reis *et al.,* 1994). The formula for N_2 estimation is:

N_2 (mg/g) = ml of H_2SO_4 in the sample x Normality of H_2SO_4 x 14.01 / Weight of the sample (Carbon used in grams)

Estimation of phytohormones: IAA and GA

The flasks containing 50 ml Czapeck's solution was prepared and autoclaved. Inoculate the flasks with 500 µl of 72 h old culture of each isolate. Incubate at 30° C for 7 days. The cultures were spinned at 3300 x g for 20 minutes. The supernatant was used for the estimation of IAA and GA. Quantitative estimation of IAA was done by Spectrophotometer method (Ivanova *et al*., 2001) and GA was estimated by the method stated by Bastian *et al*., 1998.

Phosphorus and zinc solubilization

To assess the phosphorus and zinc solubilization potential, both plate and broth assays were performed in LGI medium (Cavalcante and Dobereiner, 1988). For plate assay glucose was chosen as carbon sources at 1% concentration and medium was supplemented with insoluble zinc compounds viz., zinc oxide (ZnO), zinc carbonate (ZnCO3) and zinc phosphate (ZnPO4) as separate treatments at 0.1% concentrations. The insoluble nutrient compounds supplemented media were added to sterile Petriplates. After solidification, 48 h old ($6x10^8$ CFU mL^{-1}) cultures of *G. diazotrophicus* strains at 10 µl concentration was placed over the media and incubated at 28ºC for 3 days. After incubation, the diameter of the zone of solubilization was measured in cm. The amount of solubilization was assessed as per procedure given by Fasim *et al.*, (2002).

Antagonistic activity: Dual culture assay (Dennis and Webster, 1971)

Pathogens used:

1. *Rhizoctonia bataticola* – Charcoal rot
2. *Exserohilum maydis* – Tursicum leaf blight

The dual culture assay was done. PDA (Potato dextrose agar) was used. 5mm of mycelial disc of the pathogen was placed at the center. A loopful of the bacteria was streaked 3cm away from the pathogen on both sides and incubated at 30° C for 7 days. Petri plates inoculated with only pathogen served as control. The growth was measured using scale in centimeters.

The % growth inhibition (PGI) was calculated using the formula

PGI % = C – T/C X 100
C – growth in the control plate T – growth in the test plate

Results and Discussion

Isolation, identification and biochemical characterization of endophytic bacterial isolates

Presumptive strains of *G. diazotrophicus* were assessed in the plant samples collected from various parts of Dharwad, Belgaum, Uttar Kannada and Shimoga districts of Karnataka state in India. For preliminary screening, the cultures were isolated using LGI, a medium used for *G. diazotrophicus* isolation (Cavalcante and Dobereiner, 1988).

The formation of orange colour colony on LGI media was taken as prime criterion to identify the *G. diazotrophicus* isolates (Fig. 1) .Based on this property a total of 113 strains were identified from four crops (Table 1). 50 isolates of *G. diazotrophicus* from sugarcane, 21 from Maize, 19 from Carrot and 23 from Pineapple roots were isolated. These isolates were further characterized biochemically for specific characters of *G. diazotrophicus* according to Burgey's Manual of Systematic Bacteriology (Table 2). All the isolates were positive for most of characters specific for *G. diazotrophicus*. Further all isolates tested negative for the biochemical character like gelatin hydrolysis.

Plant growth promoting traits

The cultures were assessed for the plant growth promoting traits, such as nitrogen fixation ability, phytohormone production, phosphorus and zinc solubilization.

Nitrogen fixation ability (Microkjeldhal method)

All the 113 isolates were subjected to know the nitrogen fixation by Microkjeldhal method. 34 isolates efficiently fixed considerable amount of nitrogen. Among them ten best isolates were selected for further characterization.

An isolate from Maize GdM5fixed about 42 µg of Nitrogen/ mg of Carbon used which is equivalent to that of reference culture of *G. diazotrophicus* (MTCC1224). Whereas the isolate from sugarcane GdS25 fixed highest amount of nitrogen than other strains *i.e.*, 147 µg of N/ mg of C used. A strain from Pineapple GdP7 fixed about 49 µg of N/ mg of C. An isolate from carrot recorded lowest value of 28 µg of N/ mg of C (Table3).

Phytohormone production

IAA producing ability differed among the isolates. All the ten strains produced IAA. The highest IAA was produced by an isolate from sugarcane GdS25 i.e., 4.94 µg/ml. An isolate from carrot produced lowest IAA i.e., 3.09 µg/ml. The same trend was observed for GA production (Table 4).

Table.1 Isolates of *Gluconacetobacter diazotrophicus* from various crops

Name of the crop	Isolate recovered from	Number of isolates
Sugarcane	Root tissue	50
Maize	Root tissue	21
Pineapple	Root tissue	23
Carrot	Root tissue	19

Table.2 Selective biochemical tests of *Gluconacetobacter diazotrophicus* isolates

Particulars	*Gluconacetobacter diazotrophicus* isolates			
Crops	Sugarcane	Maize	Pineapple	Carrot
Cell Shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Gram reaction	Gram negative	Gram negative	Gram negative	Gram negative
Motility	+	+	+	+
Brown pigment on GYC medium	+	+	+	+
Gelatin liquefaction	–	–	–	–
Catalase activity	+	+	+	+
Oxidation of ethanol	+	+	+	+
Growth on C sources Glucose Sucrose Ethanol Mannitol	 + + + +	 + + + +	 + + + +	 + + + +
Growth at various concentration of sugar 5% 10% 20% 30%	 + + + +	 + + + +	 + + + +	 + + + +
Growth at various temperatures 4°C 28°C 32°C 37°C	 – + + +	 - + + +	 - + + +	 - + + +

Table.3 Nitrogen fixation ability of *Gluconacetobacter diazotrophicus* isolates by Microkjeldhal method

SI. No.	Isolate Code	µg of Nitrogen/ mg of Carbon
1	GdS25	147.10
2	GdS26	140.10
3	GdS6	91.06
4	GdS13	91.06
5	GdS24	84.06
6	GdS27	63.04
7	GdM5	42.03
8	GdM6	35.02
9	GdP7	49.03
10	GdC16	28.02
11	Reference strain of *G. diazotrophicus* MTCC1224	35.02

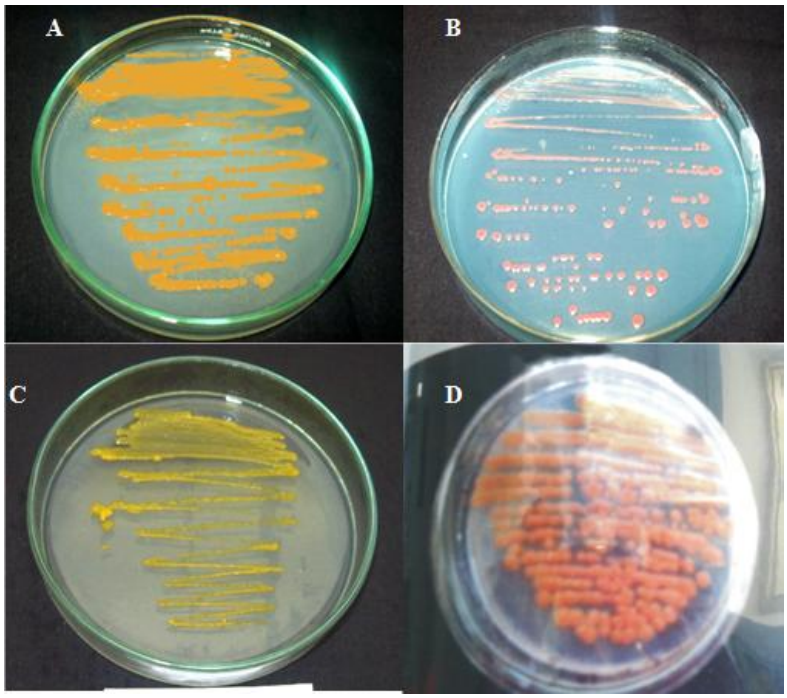
Table.4 The production of IAA, GA production, phosphorus and zinc solubilization zone by the selected efficient strains of *Gluconacetobacter diazotrophicus*

SI.No.	Isolate Code	IAA (µg/ml)	GA (µg/ 25 ml)	P solubilization zone (cm)	Zn solubilization zone (cm)
1	GdS6	4.63	5.75	1.8	2.8
2	GdS13	4.54	5.36	1.7	2.7
3	GdS24	3.96	5.18	1.5	2.2
4	GdS25	4.94	7.10	2.1	3.5
5	GdS26	4.78	6.95	2.0	3.1
6	GdS27	3.80	5.10	1.3	2.0
7	GdM5	3.42	4.54	1.4	2.6
8	GdM6	3.15	4.09	1.5	2.5
9	GdC16	3.09	3.09	1.1	1.5
10	GdP7	3.58	4.97	1.7	2.7
11	Reference strain of *G. diazotrophicus*	4.50	6.50	2.0	3.0

Table.5 The antagonistic activity of the selected efficient strains of *Gluconacetobacter diazotrophicus*

SI.No.	Isolate Code	Per cent inhibition (%) *(Rhizoctonia bataticola)*	Per cent inhibition (%) *(Exserohilum maydis)*
1	GdS6	72.23	77.78
2	GdS13	75.56	75.56
3	GdS24	74.45	80.00
4	GdS25	83.33	88.89
5	GdS26	80.00	86.25
6	GdS27	70.30	74.31
7	GdM5	77.78	83.34
8	GdM6	76.27	81.33
9	GdC16	61.12	72.28
10	GdP7	75.28	76.23
11	Reference strain of *G. diazotrophicus*	79.80	85.50

Figure.1 Colony morphologies of *Gluconacetobacter diazotrophicus* isolates A (GdS25), B (GdM5), C (GdP7) and D (GdC16) grown at 30°C for 72 h in LGI medium obtained from Sugarcane, Maize, Pineapple and Carrot, respectively



Phosphorus and zinc solubilization ability

All the ten isolates solubilized P and Zn. The larger P solubilization zone was observed in case of sugarcane isolate GdS25 i.e., 2.1cm and the lowest was in case of carrot isolate GdC16 i.e., 1.1cm.The same trend was observed for Zn solubilization ability (Table 4).

Antagonistic activity

The dual culture assay was carried out to find out the per cent inhibition. An isolate from sugarcane GdS25 exhibited 83% inhibition against *Rhizoctonia bataticola* and 88% against *Exerohilium sp.* The maize isolate GdM5 recorded 77% and 83% inhibition against both the pathogens respectively (Table 5).

Screening of *G. diazotrophicus* isolates

Out of 113 strains which were isolated from four different crops i.e., sugarcane, maize, pine apple and carrot, 5 efficient isolates viz., GdS25, GdS26, GdM5, GdM6 and GdP7 were screened based on the morphological and biochemical characterization and plant growth promoting traits.

Exploration of diazotrophs from different crops paves way to reduce the cost incurred on nitrogen fertilizers as well as minimizes the risk of pollution created by continuous application of fertilizers. Though *G. diazotrophicus* was first isolated from sugarcane (Cavalcante and Dobereiner, 1988), a further study in literature clearly showed its wide occurrence in different crops (Jimenez-Salgado *et al.,* 1997; Loganathan *et al.,* 1999; Hernandez *et al*., 2000). So, it is not astonishing to obtain 113 isolates of *G. diazotrophicus* from various crops like sugarcane, maize, pineapple and carrot. These plants contain in their cell sap considerable amount of sucrose so they can survive in the plant system and proliferate. The selective biochemical tests clearly points out the occurrence of *G. diazotrophicus*.

These putative endophytic strains of *G. diazotrophicus* also exhibited considerable amount of nitrogen fixation equivalent to that of the reference strain of *G. diazotrophicus* isolated from sugarcane environment. Among 113 isolates a strain from sugarcane (GdS25) fixed maximum amount of nitrogen and highest IAA production, indicating its possibility to be used as a bioinoculant. The endophytes are postulated to play an important role in sustainable crop production, probable mechanism of growth promotion may be by growth hormones. IAA and gibberellins have been found in the cultures of *G. diazotrophicus* (Bastian *et al.,* 1998). Till date *Azospirillum* is the promising diazotroph recommended to graminaceous crops as biofertilizer.

All the isolates exhibited significant amount of phosphate solubilizing ability and zinc-solubilizing ability. Inclusion of a bacteria solubilizing zinc, as a bioinoculant in crop production technology is really beneficial for a country like India having high incidence of zinc deficiency (more than 70%) (Alloway, 2001).

Thus, understanding basic mechanisms behind the growth promotion may help to minimize the cost of crop production e.g. phosphorus and zinc are the nutrients that are essentially required by the crops but they are relatively leached from soil or transformed into insoluble/ sparingly soluble form by complex soil reactions and become totally unavailable to plants. Hence the combined use of *G. diazotrophicus* with cheaper materials like rock phosphate and zinc insoluble compounds/ores may help in alleviating the status of these nutrients.

Apart from all these growth promotion activities, *G. diazotrophicus* strains have the additional property of biocontrol potential against soil borne pathogenic fungi and this should be explored in depth to exploit the complete potential of *G. diazotrophicus* as one of the biocontrol agents (Logeshwaran *et al*., 2011).

Based on morphological, biochemical and functional characterization, it is concluded that GdS25, GdS26 isolated from sugarcane, GdM5 , GdM6 isolated from maize and GdP7 isolated from pineapple proved to be more potent isolates and will be taken for further pot and field trials. These isolates can be exploited in future as biofertilizers for the improvement of crop productivity and as biocontrol agent against plant pathogenic fungi. The coming years are going to witness the new dimensions of plant growth promotion by *Gluconacetobacter diazotrophicus* apart from its nitrogen fixing and growth hormone producing ability. Thus the studies on *Gluconacetobacter diazotrophicus* may be diversified, several basic growth promoting properties of these bacteria can be elucidated.

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