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Characterization of endophytic nitrogen fixing bacterium *Gluconacetobacter diazotrophicus* associated with sweet potato (*Ipomoea batatas* (L.) Lam)

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Abstract: ***Gluconacetobacter diazotrophicus*, an acid tolerant diazotrophic bacterium is known to colonize the tissues of sugar rich plants and fix appreciable quantities of atmospheric nitrogen. Eighteen isolates of *Gluconacetobacter diazotrophicus* were isolated from the root, stem, leaves and tubers of sweet potato varieties grown at two different field locations (with slight different in soil pH) in the orchard of Horticultural College and Research Institute, Coimbatore. All the isolates resembled the type culture PAL 5 obtained from Brazil. They were Gram negative, motile and produced hydrogen sulphide. They failed to show oxidase and nitrate reductase enzyme activity. Among the different carbon sources, sucrose at 10% concentration was found to be the best for the better growth of the isolates. Maximum growth of G. *diazotrophicus* isolates was recorded at 30°C. Nitrogenase activity was maximum in the isolate, MT-RS-6 (236 n moles ethylene h-1 mg of cell protein-1) followed by MT-RS-16 and MT-RS-13.**

Key words : Gluconacetobacter diazotrophicus, Sweet potato, Carbon source).

Introduction

Sweet potato is a sugar rich crop next to sugarcane. The tubers of sweet potato are rich in starch and sugar and used as subsidiary food and the vines for animal feed. The crop responds well to fertilizers. Supplementing with biofertilizers, which increases the growth the tuber yield, may reduce the application of nitrogen fertilizer. The use of suitable biofertilizer to sweet potato will certainly help to boost the total tuber production and increase the net return to the farmer.

A large number of microorganisms are found to associate with the root system of sweet potato and are involved in the nitrogen and phosphorus nutrition of the crop. The beneficial role of Azospirillum (Crossman and Hill, 1987) and vesicular arbuscular mycorrhizal fungi (VAM) (Sivaprasad *et al.*, 1989) was well established. More recently, new nitrogen fixing bacterium, *Gluconacetobacter diazotrophicus* was isolated from sugar rich plants that are propagated vegetatively like sugarcane (Cavalcante and Dobereiner, 1988; Gillis *et al.* 1989), sweet potato (Paula *et al.* 1991), sweet sorghum, Cameroon grass and coffee plants (Reis *et al.* 1994; Li and MacRae, 1991; Jimenez-Salgado *et al.* 1997). Identifying a suitable better performing local strain of the organism is necessary to advocate this technology. In the present investigation an attempt was made to isolate and characterize *G. diazotrophicus* from the tissues of sweet potato and to select an efficient culture of the organism.

Materials and Methods

The sweet potato varieties CO 1, CO 2 and CO 3 were collected from the Orchard, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore at two different field locations. The isolation of *G. diazotrophicus* was carried out from the root, leaves, stem and tuber samples of the varieties. Enrichment culture technique described by Cavalcante and Dobereiner (1988) was employed. The samples were washed thoroughly with running tap water to remove the adhered soil particles, surface sterilized with 70 per cent ethanol for one minute and subsequently washed thrice with sterile distilled water. The surface sterilized samples were macerated in a sterile blender for two minutes in cold sterile cane sugar solution (5%). Serial dilutions were prepared up to 10^{-3} dilution and one ml of 10^{-3} dilution

Table 1. Characterization of *Gluconacetobacter diazotrophicus* isolates

Isolates	Gram reaction	Motility	Oxidase	Catalase	Nitrate reductase	H_2S formation	Orange colonies on acetic LGIP agar	Dark brown colonies on potato agar	Water soluble brown pigments agar	Growth at pH 3
MT-RS-1	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-2	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-3	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-4	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-5	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-6	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-7	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-8	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-9	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-10	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-11	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-12	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-13	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-14	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-15	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-16	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-17	G-ve	+	-	+	-	+	+	+	+	+
MT-RS-18	G-ve	+	-	+	-	+	+	+	+	+

+ Present
- Absent
G-ve Gram negative
GYC Glucose Yeast Extract $CaCO_3$ agar

was inoculated to various enrichment media viz., diluted cane juice semisolid medium, LGIP semisolid medium and acetic LGIP semisolid medium (Cavalcante and Dobereiner, 1988). The inoculated media were incubated at room temperature (28°C) for 72 h.

The inoculated tubes were observed for the formation of white surface pellicles in diluted cane juice medium and also for the formation of yellowish surface pellicles in LGIP and acetic LGIP semisolid media. The positive tubes were selected and the cultures were purified by streaking on to potato infusion agar medium in Petri plates (Cavalcante and Dobereiner, 1988). Discrete single colonies were sub cultured in acetic LGIP slants. The isolates were named MT-RS-1 to MT-RS 18 serially as per the sample numbers.

Gram staining of the isolates was carried out as per Hucker's modified method (Rangaswami and Bagyaraj, 1993) and the motility of the isolates was observed by hanging drop technique using a cavity slide (Aneja, 1993). Nitrate reduction test and ability of the isolates to form hydrogen sulphide were carried out following the procedures of Beishir, (1987). Catalase test (Rangaswami and Bagyaraj, 1993), oxidase

Table 2. Growth of *Gluconacetobacter diazotrophicus* isolates in different carbon sources

Isolates	Growth after 72 h of incubation (Absorbance at 620 nm)						
	Carbon source (10 per cent)						
	Source			Glucose	Cane sugar	Mannitol	Starch
	10 per cent	20 per cent	30 per cent				
MT-RS-1	0.84	0.25	0.05	0.74	0.72	0.26	0.22
MT-RS-2	0.83	0.28	0.04	0.72	0.70	0.24	0.21
MT-RS-3	0.88	0.35	0.06	0.69	0.70	0.22	0.20
MT-RS-4	0.80	0.32	0.03	0.75	0.68	0.20	0.19
MT-RS-5	0.82	0.31	0.03	0.70	0.70	0.20	0.19
MT-RS-6	0.88	0.35	0.06	0.70	0.65	0.22	0.20
MT-RS-7	0.86	0.30	0.22	0.75	0.72	0.23	0.22
MT-RS-8	0.81	0.30	0.06	0.69	0.70	0.22	0.21
MT-RS-9	0.86	0.34	0.05	0.75	0.70	0.22	0.21
MT-RS-10	0.82	0.32	0.04	0.70	0.70	0.24	0.22
MT-RS-11	0.84	0.31	0.04	0.67	0.72	0.23	0.21
MT-RS-12	0.83	0.37	0.03	0.70	0.70	0.22	0.20
MT-RS-13	0.86	0.34	0.05	0.75	0.67	0.22	0.20
MT-RS-14	0.79	0.28	0.03	0.69	0.67	0.24	0.21
MT-RS-15	0.77	0.33	0.05	0.69	0.72	0.20	0.19
MT-RS-16	0.87	0.30	0.03	0.83	0.73	0.25	0.23
MT-RS-17	0.79	0.28	0.02	0.74	0.72	0.22	0.20
MT-RS-18	0.78	0.37	0.06	0.69	0.72	0.22	0.20
PAL-5	0.91	0.34	0.04	0.90	0.89	0.26	0.22

test (Collins and Lyne, 1970), pigmentation on different agar media (Cavalcante and Dobereiner, 1988), growth on the glucose yeast extract calcium carbonate agar medium (Micales *et al.* 1985) of the isolates were performed following the standard procedures. Growth of the isolates in various carbon sources (sucrose, glucose, cane sugar, mannitol and starch all at 10% level) and in different concentrations of sucrose (10%, 20% and 30%) was determined in minimal medium (Ureta *et al.* 1995). The influence of temperature on the growth of *A.diazotrophicus* isolates was assessed by exposing to 25, 30, 35 and 40°C. The cultures were grown in controlled shaker (120 rpm) for 72h.

The acetylene reduction activity (Hardy *et al.* 1968) and the estimation of nitrogen content in the cells (Humphries, 1956) were also carried out by taking twenty ml of sterilized semisolid LGIP medium in 65 ml serum vials and added with one ml standard inoculum, mixed well and incubated for 72h. The air inside the vials was evacuated and two ml of pure acetylene gas was injected. At the time of assay 0.5 ml of gas sample was withdrawn and injected into Gas chromatograph (Nucon-Model No. 4010) and ethylene production was assayed. The nitrogenase activity was expressed as nanomoles of ethylene produced h^{-1} (mg of cell $protein^{-1}$). Protein content of cell was estimated by Lowry's method (Lowry *et al.* 1951).

$$\text{Nitrogenase activity} = \frac{\text{Height of the peak} \times \text{Volume of acetylene gas injected}}{\text{Volume of gas injected into GC} \times \text{Hours of incubation} \times \text{Milligram of cell protein}} \times 0.0006$$

The total nitrogen content in the cells of *G. diazotrophicus* was estimated (Humphries,

Table 3. Growth of *Gluconacetobacter diazotrophicus* isolates in different temperature levels

	Growth after 72 h of incubation (Absorbance at 620 nm)			
	Temperature (°C)			
Isolates	25	30	35	40
MT-RS-1	0.45	0.85	0.45	0.16
MT-RS-2	0.41	0.82	0.43	0.14
MT-RS-3	0.49	0.90	0.50	0.18
MT-RS-4	0.43	0.82	0.42	0.15
MT-RS-5	0.41	0.83	0.45	0.16
MT-RS-6	).48	0.90	0.50	0.18
MT-RS-7	).48	0.82	0.44	0.15
MT-RS-8	).46	0.82	0.42	0.12
MT-RS-9	).45	0.86	0.48	0.17
MT-RS-10	).42	0.81	0.41	0.13
MT-RS-11	).43	0.80	0.41	0.13
MT-RS-12	).40	0.78	0.39	0.11
MT-RS-13	).47	0.87	0.49	0.16
MT-RS-14	).42	0.79	0.39	0.11
MT-RS-15	).42	0.76	0.38	0.10
MT-RS-16	).47	0.88	0.48	0.17
MT-RS-17	).41	0.79	0.40	0.12
MT-RS-18	).40	0.78	0.39	0.12
PAL-5	0.50	0.91	0.51	0.19

1956). Isolated cultures were grown in acetic LGIP semisolid medium till they attained the stationary growth phase. Then ml of the medium was homogenised and digested using 10 ml of diacid mixture (con. Sulphuric acid : perchloric acid at 5:2 V/V) and made upto 50 ml with distilled water. Ten ml of the digested material was used for nitrogen determination by using Kjeltec Auto Analyser (Model No. 1030) and expressed in mg (g of C source)$^{-1}$.

Results and Discussion

G. diazotrophicus cultures were isolated from the root, leaves, stem and tubers of sweet potato grown in two different locations at the orchard, Tamil Nadu Agricultural University, Coimbatore. Totally eighteen isolates were obtained. No variation was observed between the isolates irrespective of the location or variety of sweet potato. The isolates produced whitish surface pellicle on diluted cane juice semisolid medium and yellowish surface pellicles on acetic LGIP semisolid medium exhibiting the microaerophilic nature of the organism, as that of the reference strain PAL 5 (Table 1). The results are in accordance with the findings of Cavalcante and Dobereiner (1988). The isolates were able to grow on the isolation medium, where the sucrose concentration was higher (10%) with pronounced acid production and acid tolerance as reported by Cavalcante and Dobereiner (1988) and confirmed by Boddey *et al.* (1991). Formation of yellowish surface pellicles on LGIP and acetic LGIP semisolid medium was due to the strong acid production and assimilation of bromothymol blue. As a result of this, there was a rapid decrease in pH below 3.0. This might be the reason for the inhibition of other organisms in the isolation medium and easy isolation of *G. diazotrophicus* (Li and MacRae, 1992).

The endophytic nature of this organism was proved by isolating the organism from the surface sterilized sweet potato root, stem, leaves and tubers (Reis *et al.* 1994). The organism could have colonized the root initially and lower stem epidermal surfaces and then used root tips and lateral root junctions to enter in to the plant.

Table 4. Nitrogenase activity and nitrogen content of the isolates of *Gluconacetobacter diazotrophicus*

Isolates	Nitrogenase activity*	Nirogen content in cells**
MT-RS-1	191.420	0.353
MT-RS-2	177.000	0.347
MT-RS-3	236.800	0.410
MT-RS-4	169.670	0.342
MT-RS-5	182.230	0.352
MT-RS-6	236.800	0.410
MT-RS-7	181.550	0.351
MT-RS-8	173.530	0.345
MT-RS-9	209.230	0.368
MT-RS-10	173.530	0.345
MT-RS-11	166.290	0.253
MT-RS-12	142.800	0.232
MT-RS-13	223.710	0.339
MT-RS-14	153.730	0.236
MT-RS-15	121.370	0.225
MT-RS-16	228.010	0.340
MT-RS-17	157.760	0.258
MT-RS-18	146.190	0.236
PAL-5	240.500	0.430
SEd	2.030	0.008
CD (P=0.05)	4.260	0.020

* n moles ethylene produced h^{-1} (mg of cell protein)$^{-1}$
** mg nitrogen per g cell biomass

All the isolates from sweet potato samples which produced yellowish surface pellicle on the acetic LGIP semisolid medium were characterized for *G. diazotrophicus* and compared with PAL 5 type culture. The isolates produced orange coloured colonies while the medium was decolorized, when growth on acetic LGIP medium resembling the type strain, strong acid production and assimilation of bromothymol blue were noticed. The isolates also produced chocolate brown colonies on potato infusion agar and brown water-soluble pigments on glucose yeast extract calcium carbonate agar, which are reported as important characters of *G. diazotrophicus* (Cavalcante and Dobereiner, 1988).

The present results revealed that all the isolates showed maximum growth on sucrose, glucose and can sugar when compared to other carbon sources *viz.*, mannitol and starch (Table 2). The isolates showed variation in utilization of carbon sources. The better carbon source for *A. diazotrophicus* is sucrose (Ureta *et al.* 1995). The growth of PAL 5 reference culture strain on different carbon sources had also substantiated the results of the present investigation. When the optimum sucrose level required for the isolates was tested, maximum growth was observed at 10 per cent sucrose than at 20 and 30 per cent concentration; however, growth was noticed upto 30 per cent sucrose concentration. The results indicated the ability of *A. diazotrophicus* to withstand high sugar concentration. The growth of isolate was better at 30°C when compared to 25°C, 35°C and 40°C (Table 3). The results are in agreement with the earlier reports (Cavalcante and Dobereiner, 1988; Gillis *et al.* 1989; Li and MacRae, 1991). Among the isolates, MT-RS 6, MT-Rs 13 and MT-RS 16 had recorded maximum growth at 30°C, illustrating the superiority of these cultures.

The cultures showed nitrogenase activity by acetylene reduction assay and accumulated nitrogen in the cells. However variation in the nitrogenase activity and nitrogen content

was observed among the isolates (Table 4). It might be due to genetic diversity in this endophytic bacterium as reported by Caballero-Mellada and Martinez-Romero (1994). The present findings showed that the isolate MT-RS-6 recorded higher nitrogenase activity and nitrogen content than other isolates, indicating its superiority.

From the results it is clear that the *G. diazotrophicus* isolate MT-RS-6 showed better growth and nitrogenase activity indicating the superiority and the same may be exploited for inoculation to sweet potato to derive more benefits.

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