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RESEARCH ARTICLE

SURVEY FOR THE OCCURRENCE OF *GLUCONACETOBACTER DIAZOTROPHICUS* FROM THE SUGARCANE GROWN COASTAL SALINE SOILS OF NAGAPATTINAM DISTRICT OF TAMIL NADU

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ABSTRACT

In this present study a detailed survey for the occurrence of *Gluconacetobacter diazotrophicus* populations from the Rhizosphere of sugarcane coastal saline soils of Nagapattinam district of Tamilnadu. A total number of 20 *Gluconacetobacter diazotrophicus* strains were isolated. The results of the present study also revealed a marked variation in the population of *Gluconacetobacter diazotrophicus* observed. A range of 0.58 percent to 1.15 percent of the total bacterial population was observed in the survey.

INTRODUCTION

Sugarcane is one of the major productive plant species in the world. Sugarcane is an economically important monocotyledon with a unique capacity of accumulating high amount of sucrose in its stem. Sugarcane can potentially produce approximately 45tonnes of dry weight ha^{-1} $year^{-1}$, and 22 tones' of sugar ha^{-1} $year^{-1}$. In tropical zone Maharashtra is the major sugarcane growing state covering about 9.4 lakh ha area with production where as the productivity of Tamilnadu is highest in tropical zones. Sugarcane is a renewable, natural agricultural resource because it provides sugar, besides biofuel, fiber, fertilizer and myriad of byproducts/co-products with ecological sustainability.

Gluconacetobacter diazotrophicus a gram negative acid tolerant nitrogen fixing bacteria associated with sugarcane was reported first by cavalcante and dobereiner, 1988. *Gluconacetobacter diazotrophicus* is an endophytic microorganism of the α-proteobacteria that has the capacity to fix molecular nitrogen (olena perlova *et al*., 2003). *Gluconacetobacter diazotrophicus* is a nitrogen fixing endophyte commonly isolated from Saccharaum (sugarcane). Compared to other irrigated crops are limited, hence increasing the total sugarcane production by augmenting per hectare productivity might be the viable option. *Gluconacetobacter diazotrophicus* is an endophytic diazotroph capable of nitrogen fixation, phosphorous, zinc, potassium solubilization and also producing growth promoting substances. *G.diazotrophicus* has been isolated from many sugar rich crops like sugar cane, sugar beet, sweet sorghum, ragi, pine apple, coffee and sweet potato. In addition to the above mentioned crops, some of the grasses also known to harbor. *G. diazotrophicus* in their root stem and leaves. In the present world, we are concentrating much about the pollution and its effects on soil, water and air. All the inorganic fertilizers known to cause major soil pollution, among which nitrogenous fertilizer causes varieties of soil pollution (Prabudoss 2011). Soil salinity is a serious constraint which adversely affects plant growth and development. Economic yield of plants is of great significance which is severely affected under salinity. Sugarcane is a typical glycophyte exhibiting stunted growth or no growth under salinity, with its yield falling to 50% or even more of its true potential (Subbarao and Shaw, 1985). Shrivastava *et al.* (1989) have assigned this growth suppression to the accumulation of toxic ions. Being highly crossbred, sugarcane exhibits a significant genetic variability in nature (Wahid *et al.,* 1997). Proper evaluation of this crop germplasm against salinity may prove highly fruitful venture for its successful cultivation in problem soils.

The study was conducted in Nagapattinum district of Tamilnadu state, India, which is bounded on the north by Cuddalore district, south by Palk Strait, east by Bay of Bengal and west by Thiruvarur district. The study area falls under 20 revenue villages of Nagapattinam district of Tamilnadu.

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MATERIALS AND METHODS

Survey for *Gluconacetobacter diazotrophicus* occurrence from the Rhizosphere of sugarcane

The survey was conducted at twenty locations of coastal saline soils in Nagapattinam district, Tamilnadu where sugarcane is a predominant food crop. Random selection of locations was made so that each and every sector of the experimental area would get a representation in the survey.

Details of Locations

The names of twenty locations for the survey of *Gluconacetobacter diazotrophicus* occurrence from the Rhizosphere of sugarcane (*Saccharum officinareum L.*) are given in Table I

Collection of Rhizosphere Soil Sample

In each and every location of the survey area, a field which has been under a long sugarcane monoculture practice was selected. The collection of Rhizosphere sample was made in the field having sugarcane (*Saccharum officinareum L*), as standing crop and at tillering stage of crop growth. A total number of five sugarcane plants were selected randomly at various places in the field and considered as representative sample of that location. The selected sugarcane plants were uprooted with entire root system and with the soil adhering the roots. The entire sugarcane plants together with the soil adhered to the roots were aseptically packed up in the polythene bags and transferred to the laboratory for the isolation and enumeration of *Gluconacetobacter diazotrophicus*.

Enumeration of *Gluconacetobacter diazotrophicus* Population from the Rhizosphere of Sugarcane

The sugarcane root system of a particular location, after removing large clumps of soil by gentle shaking, were collected and the soil adhering to the sugarcane roots were used to determine the population of *Gluconacetobacter diazotrophicus*. The plate count method was adopted for the determination of *Gluconacetobacter diazotrophicus* population. Ten gram of shade dried, homogenized and sieved soil was transferred to 90 ml sterile distilled water in a 250 ml Erlenmeyer flask and incubation on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension of each soil sample was subjected to tenfold dilutions upto 10^{-7} dilution. One ml of this diluted suspension was transferred aseptically to petridishes and melted LGI agar medium was poured in each petridish. Then, they were rotated in clockwise and anticlockwise direction for uniform distribution and incubated at 30°C ± 20°C for 5-7 days. After the incubation period, the *Gluconacetobacter diazotrophicus* colonies developed in each petridishes were counted using Arnold colony counter. These replications were maintained for each soil sample.

Isolation of *Gluconcetobacter diazotrophicus*

Ten gram of the air dried samples was transferred to 90 ml of sterile distilled water in a 250ml Erlenemeyer flask and incubated on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension was then diluted appropriately and 0.1 ml of the suspension was aseptically transferred into test tubes containing 10 ml of LGI semisolid medium and semisolid acetic LGI medium supplemented with yeast extract ($20mgl^{-1}$) the tubes incubated at room temperature without disturbance until the formation of sub surface pellicles.

Composition of semisolid LGI medium (Cavalcanate and Dobereiner, 1988)

	g/l
Dipotassium hydrogen phosphate	0.200
Potassium dihydrogen phosphate	0.600
Magnesium sulphate	0.200
Calcium chloride	0.020
Sodium molybdate	0.002
Ferric chloride	0.010
Bromothymol blue (0.5% solution in 0.2N KOH)	5.0 ml
Cane sugar	100.0
Agar	1.8
Distilled water	1000ml
Ph	6.0

Composition of semisolid diluted cane juice medium (Cavalcanate and Dobereiner, 1988)

	g/l
Semisolid LGI medium	250ml
Sugarcane juice	250ml
Distilled water	500ml
Agar	1.8 g

Composition of semisolid acetic LGI medium

Semisolid LGI medium was acidified with acetic acid to pH 4.5 and agar concentration was increased to 2.2 gl^{-1} according to Cavalcanate and Dobereiner (1988).

Characterization of *Gluconacetobacter diazotrophicus*

All the isolated strains of *Gluconacetobacter diazotrophicus* and reference strain PAL5 cultures were grown in acetic acid LGI medium and single colony was streaked on acetic acid LGI agar slants and the young cultures exponential phase i.e. on 7th day were taken for further characterization.

Gram staining

Gram staining was carried out as per Huker's modified method (Rangaswami and Bagyaraj, 1933).

Motility

The presence of motility in the isolated cultures was observed by hanging drop technique using cavity slide as described by Aneja (1993).

Oxidase Test

Small pieces of filter paper were soaked in 1% aqueous tetra methyl-p-phenylene diamine and placed in a petridish. Fresh young culture to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep

violet color after ten seconds indicated positive oxidase test whereas development of a light violet color indicated negative oxidase test.

Nitrate Reductase test (Beishir, 1987)

Cultures were inoculated into the test tubes containing nutrient glucose broth with one per cent KNO and incubated at 370.3°C for 48 h. Test for the presence of nitrate reductase was carried out by adding alpha napthylamine reagent to each of the nutrient broth cultures. Development of distinct red color indicated positive test and no color development indicated negative test.

Composition of nutrient glucose broth (Rangaswami and Bagyaraj, 1993)

	g/l
Glucose	5.0
Peptone	0.5
Beef extract	3.0
Sodium chloride	5.0
Distilled water	100ml

Reagents

5N Acetic acid - 294.0 ml of glacial acetic acid +706.0 ml of distilled water
Alpha napthylamine - 5.0 ml a-napthylamine + 1000 ml 5N acetic acid

Reagent

Sulfanilic reagent - 8g sulfanilic acid + 1000 ml of 5N acetic acid

Test for hydrogen sulphide formation (Beishir, 1987)

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

Composition of peptone iron broth

	g/l
Bacteriological peptone	15.0
Protease peptone	5.0
Ferric ammonium citrate	0.5
Di potassium hydrogen phosphate	1.0
Sodium thiosulfate	0.1
Distilled water	1000ml
pH	6.0

Catalase test (Rangaswami and Bagyaraj, 1993)

Loop of bacteria to be tested was taken from the solid medium and mixed with a drop of 3 per cent hydrogenperoxide on a glass slide. Catalase positive organisms showed bubbles of oxygen.

Growth on agar media

All the isolated *G. diazotrophicus* isolates were streaked on different agar media viz., LGI, Acetic LGI and Potato agar medium and the morphological characters were observed.

Composition of potato agar (Cavalcante and Dobereiner, 1988)

	g/l
Peeled potato	200.0
Sucrose	100.0
Agar	15.0
Distilled water	1000 ml
pH	5.5

* 200 g of peeled potatoes were cooked for 30 minutes in 1000 ml distilled water and the extract was used.

Growth on different concentrations of carbon substrates

Carbon sources such as sucrose and glucose were added in semisolid LGI medium at 0, 2.5, 5, 10, 15, 20 and 25 per cent concentrations. After inoculation the cultures were kept at room temperature for 7 days and the growth of the isolates was observed by the presence of yellow surface pellicle.

Growth at different pH levels

The pH of the semisolid LGI medium was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, using glacial acetic acid and above neutral pH was obtained by using KOH 7.5, 8.0, 8.5, 9.0 and 9.5 and growth of the isolates was observed after 7 days.

Studies on the growth rate of *G. diazotrophicus* in different growth media

All the twenty strains of *G. diazotrophicus* isolates were inoculated in LGI and acetic LGI broth and incubated at room temperature. The OD values were recorded at 12 h interval upto 180 h. The OD values were measured at 660 nm in spectrophotometer (Beckman DU 64).

Designation of *Gluconacetobacter* Isolates

After the characterization, *Gluconacetobacter* isolates were designated as NGDZ-1 to NGDZ-20 isolates.

RESULTS

Table 1. Survey of *Gluconacetobacter diazotropicus* occurrence in and its dessignation coastal saline sugarcane soils of *Nagapattinam* district, Tamilnadu

S.No	Location	Isolate designation
1	Kutthalam	NGDZ-1
2	Mayiladuthurai	NGDZ-2
3	Kilavelur	NGDZ-3
4	Vedaranyam	NGDZ-4
5	Nagapattinum	NGDZ-5
6	Sirkazhi	NGDZ-6
7	Tharangambadi	NGDZ-7
8	Thirukkuvalai	NGDZ-8
9	Tholuthalangudi	NGDZ-9
10	Senniyanalore	NGDZ-10
11	Thiruvalangadu	NGDZ-11
12	Mekkirimangalam	NGDZ-12
13	Thiruvaduthurai	NGDZ-13
14	Pandaravadai	NGDZ-14
15	Nallavore	NGDZ-15
16	Kokkur	NGDZ-16
17	Maruthur	NGDZ-17
18	Palaiyur	NGDZ-18
19	Paravore	NGDZ-19
20	Palayakoodalore	NGDZ-20

Table 2. Physico -Chemical Properties of Soil Samples Collected from Twenty different Locations of Coastal Saline Soils of Nagapattinam District of Tamilnadu

S.No	Location	pH	EC (dsm^{-1})	OC %	OM %	N $kgha^{-1}$	P $kgha^{-1}$	K $kgha^{-1}$
1	Kutthalam	7.65	0.58	0.23	0.39	221.90	2300	376.10
2	Mayiladuthurai	7.59	0.47	0.29	0.57	224.01	24.40	368.74
3	Kilavelur	7.40	0.41	0.39	0.67	196.00	15.77	316.00
4	Vedaranyam	7.82	0.43	0.28	0.52	220.57	15.94	238.24
5	Nagapattinum	7.70	0.30	0.29	0.46	212.78	11.91	175.84
6	Sirkazhi	7.56	0.66	0.43	0.78	197.61	10.62	296.50
7	Tharangambadi	7.48	0.56	1.51	2.57	238.70	19.60	352.54
8	Thirukkuvalai	7.70	0.59	0.36	0.62	231.12	27.74	322.99
9	Tholuthalangudi	7.50	0.60	0.20	0.44	202.05	20.00	325.00
10	Senniyanalore	8.00	0.70	0.24	0.53	187.50	12.50	412.50
11	Thiruvalangadu	7.75	0.70	0.22	0.48	230.00	17.50	350.00
12	Mekkirimangalam	7.60	0.60	0.23	0.50	130.00	25.00	325.00
13	Thiruvaduthurai	7.85	0.70	0.26	0.57	125.00	37.00	350.00
14	Pandaravadai	7.0	0.60	0.22	0.48	150.00	30.00	412.50
15	Nallavore	7.60	0.65	0.20	0.44	270.50	22.50	350.00
16	Kokkur	7.30	0.70	0.20	0.44	300.00	17.50	425.00
17	Maruthur	7.05	0.60	0.22	0.48	275.00	30.00	400.00
18	Palaiyur	7.85	0.65	0.21	0.46	262.50	35.00	362.50
19	Paravore	7.80	0.55	0.22	0.48	305.00	22.50	375.00
20	Palayakoodalore	7.50	0.65	0.23	0.50	350.00	27.50	387.50

Table 3. Occurrence of community population of *Gluconacetobacter* in twenty locations of coastal saline soils of Nagapattinam district, Tamilnadu, India

S.No	Location for soil sample collection	Log_{10} CFU/g of dry soil [Depth of collection (0-15 cm)]		
		Total Bacterial production	*Gluconacetobacter*	% of *Gluconacetobacter*
1	Kutthalam	7.83	5.62	0.62
2	Mayiladuthurai	7.75	5.57	0.67
3	Kilavelur	7.72	5.63	0.81
4	Vedaranyam	7.32	5.43	0.83
5	Nagapattinum	7.74	5.52	0.61
6	Sirkazhi	7.95	5.87	0.86
7	Tharangambadi	7.59	5.35	0.57
8	Thirukkuvalai	7.78	5.85	1.14
9	Tholuthalangudi	7.37	5.31	0.88
10	Senniyanalore	7.58	5.38	0.64
11	Thiruvalangadu	7.72	5.59	0.75
12	Mekkirimangalam	8.15	6.05	0.80
13	Thiruvaduthurai	7.67	5.53	0.68
14	Pandaravadai	7.59	5.48	0.78
15	Nallavore	7.76	5.63	0.75
16	Kokkur	7.44	5.38	0.88
17	Maruthur	8.13	6.05	0.84
18	Palaiyur	7.30	5.23	0.86
19	Paravore	7.80	5.61	0.65
20	Palayakoodalore	7.69	5.57	0.76

Table 4. General characteristics of *G. Diazotrophicus*

S.No	Characteristics	Strain behavior
1.	Gram reaction	Gram negative straight rod with round ends
2.	Pleomorphism	±
3.	Motility	Motile by 1-3 lateral flagella
4.	N_2 depended growth	N_2 fixer and grow well with combined N sources
5.	Temperature & pH	30^0C to 32^0C & 5.5
6.	Colony characters on	Small orange colored colonies
	a) LGI medium	with pellicle formation
	b) Potato infusion agar	Dark brown colonies
	c) Cyc agar	Brown colonies
7	Biochemical Characteristics	
	a) Catalase	Positive
	b) H_2S Production	Positive
	c) Oxidase	Negative
	d) Nitrate reduction	Negative
	e) Gelatin liquefication	Negative
8.	Disaccharide metabolism	Present
9.	Oxygen requirement	Microaerophilic

DISCUSSION

Muthukumarasamy *et al.* (2002) have reported Entophytes are plant associated prokaryotes that form association with their host plants by colonizing the internal tissues, which has made them valuable for agriculture as a tool in improving crop performance. They have been reported from numerous plant species including sugarcane. *Gluconacetobacter diazotrophicus* (syn. *Acetobacter diazotrophicus*) sugarcane association represents a model system for monocot diazotrophic associations. This allows experimentation to answer questions pertaining to their establishment, colonization process, and biological nitrogen fixation, growth promotion, etc. In this present study a detailed survey for the occurance of *Gluconacetobacter diazotrophicus* populations from the rhizosphere of sugarcane coastal saline soils of Nagapattinum district of Tamilnadu. A total number of 20 *Gluconacetobacter diazotrophicus* strains were isolated. The results of the present study also revealed a marked variation in the population of *Gluconacetobacter diazotrophicus* observed.

A range of 0.57 percent to 1.14 percent of the total bacterial population was observed in the survey. In the present study, twenty cultures of *Gluconacetobacter diazotrophicus* NGDZ-1 to NGDZ-20were isolated from the Rhizosphere of sugarcane coastal saline soils of Nagapattinam district of Tamilnadu were identified based upon the morphological and physiological characteristics as mentioned in Bergeys's manual of determinative Bacteriology VIII edition.

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