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RESEARCH ARTICLE

SURVEY FOR THE OCCURANCE OF *GLUCONACETOBACTER DIAZOTROPHICUS* FROM THE RHIZOSPHERE OF SUGARCANE GROWN COASTAL SALINE SOILS OF CUDDALORE DISTRICT, TAMILNADU

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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 cuddalore 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.

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INTRODUCTION

Sugarcane, the predominant agro-industrial crop in India is being cultivated about 3.8 to 5.04 million hectares with an annual production of 279 to 340 million tonnes of sugarcane in the past ten years and contributing around 7.5 per cent to the total agricultural productivity. Sugarcane production in 2009-2010 is estimated 249.48 million tonnes which is lower than the production of 273.93 million tonnes during 2008-2009. This represents a decline of 9 per cent over the previous year and 27 per cent of the targeted production for 2009-2010. Among the sugarcane growing countries India ranks first in area under production even in the problematic issues about the cost of price to the commercial cane. However, the per hectare productivity in India is comparatively lesser as compared to the countries like Brazil, Australia, Canada, Indonesia, China and Thailand. Moreover, the demand for sugar and other sweetening agents is steadily increasing year by year and the requirement has been estimated for about 625 million tonnes in 2020AD, which is around 231 per cent of the present production level.

Further the scope of expanding sugarcane cultivable area as 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 harbour *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 (Shannon, 1984; Francois, 1996). 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 & 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.

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 Cuddalore district, Tamilnadu where sugarcane is a predominant food crop. Random selection of locations were 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 five locations for the survey of *Gluconacetobacter diazotrophicus* occurrence from the

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rhizosphere of sugarcane (*Saccharum officinarum* L.) are given in Table I

Collection Of Rhizosphere Soil Sample From Each Location

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 officinarum* 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 to 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 these 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 $30 \pm 2^{\circ}$ 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 *Gluconacetobacter Diazotrophicus*

Ten gram of the air dried samples was transferred to 90 ml of sterile distilled water in a 250ml Erlenmeyer 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 (Day and Dobereiner, 1976) and semisolid acetic LGI medium supplemented with yeast extract (20mg l^{-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)	5.0ml
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	1.8g

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 g l^{-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 percent 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 colour after ten seconds indicated positive oxidase test whereas development of a light violet colour 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_3 and incubated at 37° C for 48 h. Test for the presence of nitrate reductase was carried out by adding 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.

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 naphthylamine	5.0 ml α -naphthylamine + 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
Proteose 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 hydrogen peroxide 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
	(acedified with acetic acid)

* 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 charecterization, Gluconacetobacter isolates were designated as CDZ-1 to CDZ-20 isolates.

RESULTS

Table I: Details locations for the survey of *gluconacetobacter diazotrophicus* occurrence in coastal saline sugarcane soils of cuddalore district, tamil nadu, india

S.No	Name Of District	Location
1.		Annamalai nagar
2.		Bhuvanagiri
3.		Chidambaram
4.		Cuddalore
5.		Kollidam
6.		Kuringipadi
7.		Marudur
8.		Mutlur
9.		Nellikuppam
10.	Cuddalore district	Neyveli
11.		Orathur
12.		Palur
13.		Panruti
14.		Parangipettai
15.		Pennadam
16.		Pichavaram
17.		Pinnalur
18.		Pudhuchathram
19.		Sethiathop
20.		Vadalar

Table II: Physico-chemical properties of soil samples collected from twenty locations of coastal saline soils of cuddalore district, tamil nadu, india

Location	Soil type	Organic Matter Content	pH
Annamalai nagar	Clay loam	2.25	8.0
Bhuvanagiri	Clay loam	3.02	8.0
Chidambaram	Sandy loam	2.84	6.8
Cuddalore	Clay loam	1.48	7.2
Kollidam	Clay loam	0.84	7.8
Kuringipadi	Clay loam	1.78	8.1
Marudur	Clay loam	1.56	7.3
Mutlur	Clay loam	1.36	7.2
Nellikuppam	Clay loam	0.78	8.2
Neyveli	Red soil	0.98	7.9
Orathur	Clay loam	0.92	7.4
Palur	Clay loam	1.00	8.3
Panruti	Sandy loam	1.02	8.1
Parangipettai	Sand clay	0.84	8.2
Pennadam	Clay loam	1.62	7.9
Pichavaram	Sandy loam	1.38	7.7
Pinnalur	Sandy loam	2.48	7.2
Pudhuchathram	Sandy loam	2.96	7.5
Sethiathop	Clay loam	1.56	8.0
Vadalar	Clay loam	3.12	7.4

Table III: Occurrence of community gluconacetobacter population in twenty locations of coastal saline soils of cuddalore district, tamil nadu, india

Locations for soil sample Collection	Log ₁₀ CFU/g of dry soil [Depth of collection (0-15 cm)]		
	Total Bacterial Population	Gluconacetobacter	% of Gluconacetobacter
Annamalai nagar	7.82	5.61	0.61
Bhuvanagiri	7.74	5.56	0.66
Chidambaram	7.71	5.62	0.81
Cuddalore	7.51	5.42	0.81
Kollidam	7.73	5.51	0.60
Kuringipadi	7.94	5.87	0.85
Marudur	7.58	5.34	0.57
Mutlur	7.77	5.84	1.14
Nellikuppam	7.36	5.30	0.87
Neyveli	7.57	5.37	0.63
Orathur	7.71	5.58	0.74
Palur	8.14	6.04	0.79
Panruti	7.69	5.52	0.67
Parangipettai	7.58	5.47	0.77
Pennadam	7.68	5.56	0.75
Pichavaram	7.43	5.37	0.87
Pinnalur	8.12	6.04	0.83
Pudhuchathram	7.29	5.22	0.85
Sethiathop	7.79	5.60	0.64
Vadalar	7.75	5.62	0.74

Table III: Designation of g.diazotrophicus isolated from coastal saline soil samples of twenty locations in cuddalore district, tamil nadu, india

S.No	Name Of District	Location	Isolate designation
1.		Annamalai nagar	CDZ-1
2.		Bhuvanagiri	CDZ-2
3.		Chidambaram	CDZ-3
4.		Cuddalore	CDZ-4
5.		Kollidam	CDZ-5
6.		Kuringipadi	CDZ-6
7.		Marudur	CDZ-7
8.		Mutlur	CDZ-8
9.		Nellikuppam	CDZ-9
10.	Cuddalore district	Neyveli	CDZ-10
11.		Orathur	CDZ-11
12.		Palur	CDZ-12
13.		Panruti	CDZ-13
14.		Parangipettai	CDZ-14
15.		Pennadam	CDZ-15
16.		Pichavaram	CDZ-16
17.		Pinnalur	CDZ-17
18.		Pudhuchathram	CDZ-18
19.		Sethiathop	CDZ-19
20.		Vadalar	CDZ-20

Table IV: General characteristics of g.diazotrophicus

Sl.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 Colony characters on	30 ⁰ C to 32 ⁰ C & 5.5
	a) LGI medium	Small orange colored colonies with pellicle formation
6.	b) Potato infusion agar	Dark brown colonies
	c) Cyc agar	Brown colonies
	Biochemical Characteristics	
	a) Catalase	Positive
	b) H ₂ S Production	Positive
7.	c) Oxidase	Negative
	d) Nitrate reduction	Negative
	e) Gelatin liquefaction	Negative
8.	Disaccharide metabolism	Present
9.	Oxygen requirement	Microaerophilic

DISCUSSION

R. Muthukumarasamy *et al.*, (2002) have reported Endophytes 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, biological nitrogen fixation, growth promotion, etc. In this present study a detailed survey for the occurrence of *Gluconacetobacter diazotrophicus* populations from the rhizosphere of sugarcane coastal saline soils of cuddalore 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* CDZ-1 to CDZ-20 were isolated from the rhizosphere of sugarcane coastal saline soils of cuddalore 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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