ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 3, Issue, 6, pp.103-106, June, 2011

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

A REAL MULTI BENEFICIAL ENDOPHYTIC DIAZOTROPH *Gluconacetobacter diazotrophicus* FOR SUGARCANE

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ARTICLE INFO

Article History:
Received 9th February, 2011
Received in revised form
15th April, 2011
Accepted 27th May, 2011
Published online 2nd June 2011

Key words:
Gluconacetobacter diazotrophicus,
Sugarcane,
Endophytes,
Diazotrophs,
Pollution,
Fertilizers.

ABSTRACT

Gluconacetobacter diazotrophicus, a true fruitful, sugar loving and acid producing endophytic diazotroph involved in N fixation, P solubilization, zinc solubilization and growth promoting substance production. It can also able to control fungal pathogens likes *fusarium* and *Colletotrichum falcatum* and helps the sugarcane plants to produce its maximum growth with the reduction of 50 per cent chemical fertilizers. The present field experiment was laid out with the following treatments. T_0 - Absolute control, T_1 - *G. diazotrophicus* alone, T_2 - 50% NPK alone, T_3 - 50% NPK + *G. diazotrophicus,* T_4 - 75% NPK alone, T_5 - 75% NPK + *G. diazotrophicus,* T_6 - 100% NPK alone, T_7 - 100% NPK + *G. diazotrophicus* and three replications were also maintained in the field studies. Among the eight treatments, the plants receiving *G. diazotrophicus* inoculation showed the vigour in the growth and developments of sugarcane, particularly the treatments. T_3 - 50% NPK + *G. diazotrophicus* and T_5 - 75% NPK + *G. diazotrophicus* recorded the growth values on par with the growth values of plants receiving 100% NPK + *G. diazotrophicus* (T_7) and T_6 - 100% NPK alone. Hence, by using *G. diazotrophicus* we can able to reduce 50% usage of chemical fertilizers and it leads to reduce the pollution to some extent in sugarcane cultivating soil and environment.

INTRODUCTION

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. The crop sugarcane consumes large amount of nitrogen fertilizers because of long standing period in the field, hence a alternative way is needed to supply the nutrients in adequate amount without causing any deleterious effect on living things in soil and in the environment. Sugarcane is a well suited internal system for invading *G. diazotrophicus* and the conditions prevailing in the tissues of sugarcane has been a wonderful site for the growth and development of *G. diazotrophicus*. It fixes appreciable amount of nitrogen in the endorhizosphere, endocaulosphere and endophyllosphere and produces growth promoting substances in the above mentioned sites of sugarcane and also it can restrict the growth of pathogens like *Fusarium* and *Colletotrichum falcatum* by synthesizing compounds like Pyrrolnitrin and Pyoluteorin. Recent findings in sugarcane research specifically to nitrogen nutrition have brought proof of more than 50 percent nitrogen and some time exceeds 70 percent of the plant nitrogen coming from air through biological nitrogen fixation (Boddey *et al.,* 2003). The country like Brazil cultivating sugarcane crop with minimized use of chemical fertilizers or without using chemical fertilizers and it shows indirectly presence of reserved nutrients in the soil, yes not in the form of nutrients but in the form of effective multi-beneficial microorganisms, among which *G. diazotrophicus* has been well exploited for the potentiality to improve nutrients like N, P and K in many ways and hence we have selected this naturally effective endophyte for our studies. Based on the above views, the field experiment was carried out to exploit the potentiality of *G. diazotrophicus* in different environmental field conditions with the treatments of *G. diazotrophicus* along with graded levels of N, P and K fertilizers at the rate 50%, 75% and 100% and control was also maintained.

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MATERIALS AND METHODS

The glassware's and chemicals used for the isolation studies in pure and in aseptic condition. The sugarcane samples like root, bud and leaves were collected from different places from

Cuddalore District, Tamilnadu, India namely Annamalainagar, Vilagam, Arasur, Cuddalore and Vallampadugai were used for the isolation of *G. diazotrophicus*.

Isolation of *G. diazotrophicus:* *G. diazotrophicus* cultures were isolated from the sugarcane samples following the methodology of Cavalcante and Dobereiner (1988). One gram of the sugarcane samples (root/ stem/bud/leaf) were washed thoroughly in running tap water placed in 70 per cent alcohol for 15 seconds and immediately washed in sterile distilled water for 3-4 times repeatedly. The surface sterilized samples were macerated in a sterile pestle and mortar. A drop of the suspension as such and 10^{-2} dilution of it was inoculated into different enrichment media viz., semisolid LGI, acetic LGI and diluted cane juice medium with the following composition and with slight alteration in the case and acetic LGI and diluted cane juice medium, 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, then the tubes were incubated at room temperature without disturbance until the formation of sub surface pellicles.

Characterization of *G. diazotrophicus:* All the isolated strains of *G. diazotrophicus* and reference strain PAL5 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 gram staining. Gram staining was carried out as per Huker's modified method (Rangaswami and Bagyaraj, 1933).

Screening of isolates for nitrogen fixation: The nitrogen fixing capacity of the test organisms was evaluated by using Acetylene Reduction Activity (ARA) following the standard procedure (Bergensen, 1980). Twenty five ml of semisolid LGI medium was prepared in 100 ml vials. The vials were inoculated with 25 µl of *G. diazotrophicus* isolates and incubated under static condition in an incubator 28 ± 1°C. After 5 days of growth the cotton plugs were replaced by suba-seal septa and tightened with aluminium cap. The air in the vial was replaced with nitrogen gas. Ten per cent (v/v) of the inert gas was removed and 10 per cent pure acetylene gas was injected. The vials were incubated for 24 h at room temperature. After incubation, 1 ml of gas sample was withdrawn and injected into the gas chromatograph (Systronics 4010, India) fitted with porapak Q column (6″ × 1/8″) and FID detector. The column temperature was maintained at 80°C. Nitrogen gas was used as carrier gas at the flow rate of 20ml min^{-1}. The acetylene reduction activity of the strains was calculated using the formula:

$$\frac{\text{Sample peak length of ethylene (mm)} \times \text{Attenuation} \times \text{Volume of gas phase of flask} \times 0.0006}{\text{Incubation time (h)} \times \text{Volume of gas simple injected into gas chromatograph (ml)}}$$

The acetylene reduction activity of the sample was expressed as n moles of ethylene formed mg of protein h^{-1}. At the end of experimental period the cell protein content of the cultures were determined following the method described by Lowry *et al.* (1951).

Growth and yield of sugarcane

Shoot length and root length of sugarcane was observed at monthly intervals were measured and expressed in cm. The total number of milleable canes was counted and expressed in 000 ha^{-1}, girth of canes was measured at the time of harvest and average recorded as girth of cane in mm, at the time of harvest individual can weight were taken and expressed in kg and cane harvested from the sub plots were weighed and expressed as t ha^{-1}.

RESULTS

Isolation, characterization and nitrogen fixing efficiency of *G. diazotrophicus*

G. diazotrophicus was isolated from sugarcane samples collected from the five locations and four different parts namely root, stem, bud and leaves of sugarcane used *G. diazotrophicus* was isolated from all the parts of CoC 92061, Cosi 98071 Co 8208 and Co 86032. Twenty isolates were obtained from different parts of sugarcane and the formation of yellow coloured sub surface pellicle were observed on colourless semisolid LGI medium and typical heavy orange yellow coloured subsurface pellicle on acetic LGI semisolid medium (Table 1). The twenty isolates and the reference strain PAL5 of *G. diazotrophicus* stains were confirmed by performing characterization test, particularly gram reaction. All the twenty isolates were gram negative since they developed red coloured cells after gram reaction. When observed under phase contrast microscope, they were rod shaped with rounded ends. All the twenty isolates and reference strain PAL5 recorded nitrogenase activity. The maximum of 386.20 n moles of C_2H_4/hr/mg cell protein was showed by *G. diazotrophicus* GdVSB* followed by *G. diazotrophicus* GdVSB (382.10 n moles C_2H_4/hr/mg cell protein). Other isolates were recorded intermediate values in ARA activities (Table 2).

Inoculation effect of *G. diazotrophicus* on the growth and yield of sugarcane

In general, the root length was significantly higher in treated plants than in control. Those plants receiving 50 per cent NPK + *G. diazotrophicus* showed higher root length (39.8) followed by 75 per cent NPK + *G. diazotrophicus* treated plants (36.9), as compared with control. Maximum shoot length was recorded by sugarcane plants receiving 50 per cent NPK + *G. diazotrophicus* (365.0 cm), followed by 75 per cent NPK + *G. diazotrophicus* (341.2 cm) during 10 month. Significant increase was seen due to treatment of *G. diazotrophicus*, with 50 and 75% NPK application and recorded best growth of shoot length (Table 3). There was significant difference in the milleable can count among the treatments, 50 per cent NPK + *G. diazotrophicus* recorded increased number of milleable canes (106.86 '000 ha^{-1}) followed by 75 per cent NPK + *G. diazotrophicus* (105.91 '000 ha^{-1}) over control (66.80 '000 ha^{-1}). The treatment receiving 50 per cent NPK + *G. diazotrophicus* (1.162 kg) and 75 per cent NPK + *G. diazotrophicus* (1.159 kg) showed an increase over 100 per cent N control (1.156 kg). A significant increase was observed in the treatments receiving 50 and 75 per cent NPK + *G. diazotrophicus* than other treatments and control. Maximum cane yield was noticed in 50 per cent NPK + *G. diazotrophicus* treatment (119.19 t ha^{-1}) followed by 75

Table 1. Isolation of *G. diazotrophicus* from different parts of sugarcane growing in different locations of Cuddalore District, Tamilnadu

S.No.	Location	Sample	Name of the varieties	Medium used			Name of the isolates	Gram reaction	Shape
				LGI	ALGI	DCJM			
1	Annamalainagar	Sugarcane root	COC 92061	+	++	+	GdASR	Negative	Rod shaped
2	Annamalainagar	Sugarcane stem		+	++	+	GdASS	Negative	Rod shaped
3	Annamalainagar	Sugarcane bud		+	++	+	GdASB	Negative	Rod shaped
4	Annamalainagar	Sugarcane leaves		+	++	+	GdASL	Negative	Rod shaped
5	Vilagam	Sugarcane root	COSi 98071	+	++	+	GdVSR	Negative	Rod shaped
6	Vilagam	Sugarcane stem		+	++	+	GdVSS	Negative	Rod shaped
7	Vilagam	Sugarcane bud		+	++	+	GdVSB	Negative	Rod shaped
8	Vilagam	Sugarcane leaves		+	++	+	GdVSL	Negative	Rod shaped
9	Arasur	Sugarcane root	CO 8208	+	++	+	GdASR*	Negative	Rod shaped
10	Arasur	Sugarcane stem		+	++	+	GdASS*	Negative	Rod shaped
11	Arasur	Sugarcane bud		+	++	+	GdASB*	Negative	Rod shaped
12	Arasur	Sugarcane leaves		+	++	+	GdASL*	Negative	Rod shaped
13	Cuddalore	Sugarcane root	CO 86032	+	++	+	GdCSR	Negative	Rod shaped
14	Cuddalore	Sugarcane stem		+	++	+	GdCSS	Negative	Rod shaped
15	Cuddalore	Sugarcane bud		+	++	+	GdCSB	Negative	Rod shaped
16	Cuddalore	Sugarcane leaves		+	++	+	GdCSL	Negative	Rod shaped
17	Vallampadugai	Sugarcane root	COSi 98071	+	++	+	GdVSR*	Negative	Rod shaped
18	Vallampadugai	Sugarcane stem		+	++	+	GdVSS*	Negative	Rod shaped
19	Vallampadugai	Sugarcane bud		+	++	+	GdVSB*	Negative	Rod shaped
20	Vallampadugai	Sugarcane leaves		+	++	+	GdVSL*	Negative	Rod shaped

GdA – *G. diazotrophicus* isolates, Annamalainagar; + - Moderate growth; GdV– *G. diazotrophicus* isolates from Vilagam ++ - Good growth; GdA*– Strains from Arasur; Gdc – Strains from Cuddalore; GdV* – Strains from Vallampadugai; ALGI – Acetic LGI medium; DCJM – Dilute cane juice medium

Table 2. Nitrogen fixing efficiency of *G. diazotrophicus* isolates from different parts of sugarcane

S.No.	Name of the isolates	Nitrogenase activity (n moles C_2H_4/hr/mg cell protein)
1	GdASR	345.90
2	GdASS	373.93
3	GdASB	380.96
4	GdASL	208.10
5	GdVSR	346.92
6	GdVSS	374.96
7	GdVSB	382.10
8	GdVSL	210.21
9	GdASR*	338.96
10	GdASS*	368.90
11	GdASB*	375.94
12	GdASL*	210.39
13	GdCSR	340.97
14	GdCSS	370.91
15	GdCSB	379.12
16	GdCSL	205.32
17	GdVSR*	350.98
18	GdVSS*	378.62
19	GdVSB*	386.20
20	GdVSL*	230.10
21	PAL5 (Reference strain)	360.20
SE		2.84
CD (p = 0.05)		6.02

per cent NPK + *G. diazotrophicus* treatment it was par with 100 per cent NPK treatments (117.08 t ha^{-1}) the values are tabulated in Table 2.

DISCUSSION

G. diazotrophicus can able colonize almost all types sugarcane varieties cultivating in India. Out of the four varieties of sugarcane used, *G. diazotrophicus* was isolated from all the four varieties. If the absence of this diazotroph in certain varieties may be due to high N and P fertilization of these varieties with the report made by Li and Mac Rae (1991), Fuentes – Ramirez *et al.* (1993) and Baldani *et al.* (1997). The isolates were isolated from all parts of sugarcane viz., root, stem, bud and leaf (Cavalcante and Dobereiner, 1988). More isolates were obtained when the macerates of surface sterilized root, stem, bud and leaves of sugarcane were used than the plant bits as such. This observation is substantiated by Dong *et al.* (1994) and James *et al.* (1994). This observation illustrated the endophytic nature of this bacterium (Reis *et al.* 1994; dos Santos *et al.,* 2010). It is the only nitrogen fixing bacterial endophyte and leading to designated as a model organism to evaluate the plant bacterial interactions in non-legumes (Dobbelaere *et al.,* 2003). The isolates were gram negative and

Table 3. Effect of *G. diazotrophicus* and graded levels of NPK fertilizers on the growth and yield of sugarcane

	Treatment	Root length (cm)	Shoot length (cm)	Milleable canes (000 ha^{-1})	Cane girth (cm)	Individual cane weight (kg)	Cane yield (t ha^{-1})
T_0	Absolute control	30.6	229.89	66.80	69.80	0.750	56.33
T_1	*G. diazotrophicus* alone	33.5	293.1	89.74	76.31	0.849	76.18
T_2	50% NPK alone	33.7	314.3	96.18	80.60	0.931	90.79
T_3	50% NPK + *G. diazotrophicus*	39.8	365.0	106.86	89.99	1.162	119.19
T_4	75% NPK alone	34.9	330.0	94.44	83.10	0.937	88.49
T_5	75% NPK + *G. diazotrophicus*	36.9	341.2	105.91	89.60	1.159	117.10
T_6	100% NPK	36.8	336.7	105.82	89.58	1.158	117.00
T_7	100% NPK + *G. diazotrophicus*	36.9	337.0	105.90	89.60	1.158	117.08
	SE	1.31	1.66	0.16	0.09	0.02	0.27
	CD (p = 0.05)	2.64	3.77	0.36	0.17	0.05	0.58

rod shaped as described by Cavalcante and Dobereiner (1988). The nitrogenase activity of *G. diazotrophicus* strains was first proved by Cavalcante and Dobereiner (1988) in a report indicating 240 n moles C_2H_4 h^{-1} mg cell $protein^{-1}$ and later confirmed by Gillis *et al.* (1989). In the present study, all the isolates showed appreciable amounts of nitrogenase activity ranging from 205.32 to 386.20 n mole $C_2H_4h^{-1}$ mg cell $protein^{-1}$. The variation may be due to collection of sugarcane samples from different locations and different varieties. The same was also reported by Caballero-Mellado and Martinez-Romera (1994); Oliveira *et al.* (2009). Regarding the growth parameters like root length, shoot length, milleable cane, cane girth, individual cane weight and cane yield the *G. diazotrophicus* and 50, 75 per cent N and P treated plants performed better than the control. Thangaraju and Jayakumar (2002) and Muthukumarasamy *et al.* (1999) have recorded can height and cane yield of 50 per cent N and P + *G. diazotrophicus* on par with 100 per cent N and P treatment. In the present study, *G. diazotrophicus* treatment in combination with 50 per cent inorganic NPK fertilizers registered more number of milleable cane, cane girth and cane yield than 75 and 100 per cent NPK treatment. Inoculation of *G. diazotrophicus* could enhance the cane height, cane girth and cane yield. Hence possibility of saving 25 to 50 per cent of fertilizer NPK through the inoculation of *G. diazotrophicus* was reported by Thangaraju and Govindarajan (2001).

Summary and conclusion

The plants receiving 50% NPK + *G. diazotrophicus* and 75% NPK+*G. diazotrophicus* recorded the growth parameters values on par with the plants receiving recommended dose (100%) of NPK fertilizers from different treatments. A significant increase in the growth parameters in the form of root length, shoot length, cane girth, individual cane weight and cane yield was noticed in the treatments receiving 50% NPK and 75% NPK along with *G. diazotrophicus* compared with control. Based on the above findings, it is clear that *G. diazotrophicus* promotes sugarcane yield with the reduction of 50% NPK fertilizers and paves way for the reduction 50% chemical fertilizers in sugarcane cultivation as well as reduction of 50 per cent cost of cultivation which incurred in fertilizer usage.

Future work

There are lots of chances for cultivating other sugar rich crops by using *G. diazotrophicus* as a biofertilizer and hence we need to exploit the potentiality of these diazotrophs to other sugar rich crops, as well as cereal crops.

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