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Effects of *Gluconacetobacter diazotrophicus* on seed cane produced through micro-propagated plantlets in sugarcane (*Saccharum* spp hybrid complex) under sub-tropics

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ABSTRACT

Field experiment was conducted to evaluate the performance of micropropagated plantlets inoculated *in vitro* with *Gluconacetobacter diazotrophicus* on the population counts of diazotroph, plant growth, yield, quality and ultimately planting material of sugarcane cultivar CoS 96268. Maximum cell counts (5.8×10^5 cells/g fresh weight) was obtained in the *in vitro,* treated micropropagated plantlets-followed by untreated micropropagated plantlets (4.7×10^4 cells/g fresh weight) while it was only 3.7 $x10^4$ cells/g fresh weight in conventionally grown plants. The highest cell counts of *Gluconacetobacter diazotrophicus* was recorded at grand growth stage which decreased at maturity. Inoculation with diazotroph improved dry matter accumulation specially in roots accompanied by higher uptake of nitrogen and potassium in micropropagated plantlets. There was significant increase in number of millable canes, cane length, number of nodes, cane diameter and cane yield. Besides, treated plantlets produced 5.45 and 1.52 times higher planting material (3 budded setts) over conventionally grown plants and un-treated micropropagated plantlets, respectively. Results signify the use of inoculation with diazotrophic bacteria in micropropagated plantlets for faster multiplication of disease-free healthy seed cane of sugarcane varieties for their adoption.

Key words: Biological nitrogen fixation, Dry matter partitioning, *Gluconacetobacter diazotrophicus,* Micropropagated plantlets, Seed cane, Sugarcane

Sugarcane (*Saccharum* spp. hybrid complex) is one of the most important tropical C_4 crops, which is cultivated in tropical and sub-tropical areas throughout the world contributing 75% to the global sugar production (FAOSTAT 2011). Continued efforts are being made by the plant breeders to develop new high-yielding varieties of sugarcane with improved quality traits, which alone contributes about 10–15% increase in the production. Moreover, good quality seed cane is another important input as it increases production by 15 to 20% (Ramanand and Lal 2004). The seed multiplication ratio in sugarcane through conventional method is quite low (1: 10/year), meaning thereby that it takes about 10 years to spread a new variety over large area (Pawar *et al.* 2002). The production of quality seed cane through micropropagation technique is now well recognized in sugarcane (Tarique *et al.* 2010) and seed cane produced through this technique is considered as breeder seed in 3-tier system of seed cane production.

Sugarcane, being a huge biomass accumulating crop, demands high nitrogen nutrition. It is in this context that nitrogen-fixing bacteria have played a major role in sugarcane production. It is estimated by using ^{15}N-isotopes balance and abundance studies that more than 60% of the N-uptake (150 kg N/ha/year) was through biological nitrogen fixation (BNF) in sugarcane varieties and even some sugarcane varieties have been found to derive up to 70% of their nitrogen requirement through this mechanism (Boddey *et al.* 2001). Among the microbial pool, *Gluconacetobacter diazotrophicus* is recognized for substantial contribution to nitrogen nutrition in sugarcane as reported by Suman *et al.* (2001) and they have also reported seven isolates of *G. diazotrophicus* from the sugarcane varieties grown in subtropical India. Of these isolates, IS 100 was found most effective to increase sugarcane productivity in sub-tropical India. For any beneficial microbe to benefit the plant, the indigenous isolates have advantage

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in establishing in local environment and competing with the native flora (Suman *et al.* 2005). Though the most convincing advantage presented by the micropropagation process is the clonal cleaning from pathogenic bacteria and fungi, but it also eliminates all the beneficial endophytic microorganisms that could promote plant growth. It provides an opportunity for *in vitro* inoculation of efficient isolate IS 100 of *G. diazotrophicus* to increase the sugarcane productivity. The feasibility of the inoculation technology using diazotrophic bacteria in micropropagated plantlets for improvement of total nitrogen accumulation and yield was reported in Brazil (Oliveira *et al.* 2002, 2006). Keeping this in view, the present study aimed at evaluating the performance of micropropagated plantlets inoculated *in vitro* with IS 100 isolate of *G diazotrophicus* in comparison to un-inoculated plantlets and conventional ones for their growth, yield and quality as well as seed multiplication ratio of sugarcane variety in sub-tropical India.

MATERIALS AND METHODS

Micropropagated plantlets were produced from apical meristem of the commercially important sugarcane cultivar, CoS 96268 during 2008–09 using modified MS solid and liquid media under aseptic growth conditions (Murashige and Skoog 1962). Fully developed plantlets were obtained in 120 days. MS medium with modification in the concentration of growth hormones was used for shoot initiation (MS basal + 1.0 g/l BAP + 1.0 g/l kinetin + 3.0% sucrose + 8.0 g/l agar), multiplication of shoot (MS basal + 2.0 g/l BAP + 2.0 g/l kinetin + 0.5 g/l NAA+ 0.5 g/l GA_3 + 3.0% sucrose) and development of root ($^1/_2$ MS basal + 5.0 g/l NAA+ 6.0% sucrose) of plantlets. Hardening of plantlets was carried out in the soil mixture (1 soil: 1 compost: 1 sand) with more than 80% humidity.

Uniform plantlets with fully developed roots were individually transferred to flasks containing 15 ml of modified MS medium (Singh *et al*. 2009), Three days after transfer, only those flasks with no apparent contamination were taken for inoculation with IS 100 isolate of *G. diazotrophicus*. The plantlets with intact and incised roots were dipped in *G. diazotrophicus* suspension (4.7×10^8 cells/ml) for 10 min. The treated plantlets were transferred in the new medium under aseptic conditions. Un-inoculated plantlets of intact and incised root plantlets were maintained separately. Five replicates (flasks) each containing 10 plantlets were used for sampling each time. Treated plantlets were maintained at 28 ^oC with 60 $\mu mol/m^2/s^2$ illuminance for 14 hr/day. After seven days of inoculation, sampled roots were analyzed for *G. diazotrophicus* colonization by light microscopy using Leica phase contrast microscope after confirmation of bacterial presence. The plantlets were subjected to hardening as described by Singh *et al.* (2009).

The treated and hardened plantlets were transplanted in field along with un-inoculated plantlets and conventionally grown plants of variety CoS 96268 in single row of 6m long keeping plant-to-plant distance of 30 cm in seven replications in randomized block design. The experiment was conducted at Indian Institute of Sugarcane Research, Lucknow during 2008–09, located at $26^056'$N latitudes, 80^0 52' E longitudes and 111 m above mean sea level, having semi-arid climate with hot summers and fairly cool winters in subtropical belt of India. The recommended package of practices was followed for raising the crop.

Five sugarcane plants from each treatment (inoculated plantlets, un-inoculated plantlets and conventionally grown) were uprooted with intact root system at grand growth stage (mid August) and at maturity stage (mid February). Cell counts of *Gluconacetobacter diazotrophicus* at different stages of sugarcane growth were measured using the methods reported by Suman *et al.* (2007). Bacterial cell count was calculated using MPN-McCardy Tables and presented in Table 1.

Table1 Cell counts of *Gluconacetobacter diazotrophicus* at different stages of sugarcane growth

Treatment	Grand growth stage	Maturity stage
Inoculated plantlets	5.8×10^5	5.4×10^5
Un-inoculated plantlets	4.7×10^4	3.9×10^3
Conventionally grown plants	3.7×10^4	3.0×10^3

At the harvest of the crop, five plants were uprooted randomly from each plot with intact root system by digging into 50 cm depth to avoid damage to the roots for N, P and K determination as reported by Singh *et al*. (2007). Observations on number of millable canes (NMC), number of nodes, stalk diameter (cm), stalk length (cm), single stalk weight (kg), cane yield/clump (kg) and number of three budded setts/clump were recorded at 12 month crop age. Among quality traits, the brix was measured at this stage. The data on these traits were subjected to standard statistical analyses.

RESULTS AND DISCUSSION

Enumeration of cell counts of Gluconacetobacter diazotrophicus

The enumeration of G. *diazotrophicus* in terms of cell counts was observed at grand growth stage (mid August) and at maturity stage (mid February) in the roots (Table 1). At grand growth stage, the maximum cell counts was obtained in the *in vitro* treated micropropagated plantlets (5.8×10^5 cells/g fresh weight), followed by untreated micropropagated plantlets (4.7×10^4 cells/g fresh weight) against only 3.7×10^4 cells/g fresh weight in conventionally grown plants. However, at maturity the cell counts of *G. diazotrophicus* decreased to 5.4×10^5, 3.9×10^3 and 3.0×10^3 cells/g fresh

weight in corresponding treatments. Presence of maximum number of cells of *G. diazotrophicus* in *in vitro* treated micropropagated plantlets was obvious as IS 100 strain inoculated roots before hardening (Singh *et al.* 2009) got sufficient time for colonization and acclimatization in the rhizospheric ecosystem. It also confirms the earlier reports that among the available endophytes of sugarcane, *G. diazotrophicus* contributes substantially to nitrogen nutrition of the plant by better colonization (Suman *et al.* 2005). Thus, insertion of nitrogen-fixing bacteria in the micropropagated plants minimizes the competition between inoculated microbe and the native community during the initial stage of root colonization thereby the indigenous isolates have an edge over in establishing in local environment and competing with the native flora (Suman *et al.* 2001, 2005). Reis *et al.* (1999) have shown that an incubation period of seven days favours the infection and establishment of the inoculated bacteria inside the plant tissues of the micropropagated plantlets in sugarcane. There was an increase in the population of *G.diazotrophicus* in the inoculated plantlets over un-inoculated ones possibly due to high degree of colonization by the inoculated bacteria. Similarly, the population of *G.diazotrophicus* in un-inoculated micropropagated plantlets was also higher in comparison to conventionally grown plants as a result of natural colonization of such bacterium in micropropagated plants just after acclimatization period (Oliveira *et al.* 2002). There was reduction in the cell counts of *G. diazotrophicus* at maturity irrespective of treatments (Table 1). It is inferred earlier that the population of BNF are influenced by nutritional status of the plant, including the nitrogen level (Reis *et al.* 2000), tissue utilized and age of plants (Fuentes-Ramirez *et al.* 1999). The findings corroborate with those of Oliveira *et al*. (2002) who could not detect the BNF population in sugarcane plants harvested after 400 days of inoculation.

Dry matter partitioning

Data on dry matter partitioning of sugarcane plants (Table 2) clearly indicate that *in vitro* endophytic diazotrophs treated micropropagated plants accumulated higher root biomass. However, the dry matter produced by other plant parts were almost similar to that of untreated plants. On the other hand, the untreated micropropagated plants produced higher dry matter as compared to control treatment. At grand growth stage, the dry matter accumulation in stalk under control was higher (29.00%) than in treated sugarcane plants. Under control treatment early stalk formation in sugarcane plant resulted in early maturity producing low cane yield. On the other hand, micropropagated plants exhibited longer leaf area duration (higher green leaves) with prolonged elongation phase resulting into higher yield. The total dry matter produced by stalk under microbial treated sugarcane at maturity was observed to be higher (555.50 g/clump). However, the data on dry matter partitioning clearly indicate that its distribution towards the root and green leaves was higher under treated plantlets resulting into production of thicker and succulent canes as compared to un-inoculated plantlets. These are the desirable traits for higher sugar recovery to combat with the problem of longer kill to mill period.

N P K uptake

Observations on NPK uptake recorded at grand growth and maturity stages (Table 3) clearly indicate that the uptake of nitrogen and potassium was higher under *diazotropicus* treated micropropagated sugarcane plantlets. However, the uptake of P was slightly higher in un-inoculated micropropagated sugarcane plantlets may be due to the comparative lower *p*H content (Serralta *et al.* 2006). The higher uptake of N and K in sugarcane is due to higher dry matter accumulation under the treatment as compared to control as the uptake of nutrients in the plant is mainly the function of dry matter produced and its nutrient content. In the studies of Oliveira *et al*. (2002) around 30% of total nitrogen accumulation occurred in micropropagated plantlets inoculated with the mixture of strains of endophytic *diazotrophs*.

Table 2 Dry matter partitioning at different growth stages of sugarcane under three treatments

Treatment	Total dry matter (g/clump)									
	Grand growth stage					Maturity stage				
	Root	Green leaf	Dry leaf	Stalk	Total	Root	Green leaf	Dry leaf	Stalk	Total
Inoculated plantlets	23.83	57.41	16.15	33.2	130.59	(118.90)	168.3	148.5	555.5	991.2
	(18.25)	(43.96)	(12.37)	(25.42)	(100.00)	(12.00)	(16.98)	(14.98)	(56.04)	(100)
Un- inoculated plantlets	7.75	76.39	15.17	30.63	129.94	98.7	135.36	114.9	419.04	768
	(5.96)	(58.79)	(11.68)	(23.57)	(100)	(12.85)	(17.63)	(14.96)	(54.56)	(100)
Conventionally-grown	9.4	50.07	15.27	30.72	105.91	83.25	106.68	102.24	409.63	701.8
plants	(8.88)	(47.7)	(14.42)	(29)	(100)	(11.86)	(15.2)	(14.57)	(58.37)	(100)
CD (*P*=0.05)	0.677	5.175	ns	2.069	4.921	5.099	5.509	3.666	16.094	31.321
CV (%)	4.25	7.25	7.34	5.64	3.46	4.36	3.46	2.58	2.99	3.28

Dry matter partitioning (%) values are in parentheses

Table 3 NPK uptake (g per clump) by different plant parts of sugarcane under three treatments at grand growth and maturity stages

Treatment		NPK uptake									
		Grand growth stage					Maturity stage				
		Root	Green leaf	Dry leaf	Stalk	Total	Root	Green leaf	Dry leaf	Stalk	Total
N	Inoculated plantlets	0.195	0.615	0.142	0.315	1.267	0.547	0.909	0.876	3.555	5.887
	Un-inoculated plantlets	0.052	0.527	0.061	0.214	0.854	0.365	0.596	0.414	2.263	3.637
	Conventionally-grown plants	0.051	0.354	0.06	0.181	0.646	0.291	0.395	0.296	1.639	2.621
	CD (*P*=0.05)	0.004	0.017	0.006	0.025	0.068	0.014	0.02	0.017	0.101	0.145
	CV (%)	3.68	2.86	5.96	9.03	6.37	3.03	2.7	2.7	3.49	3.08
P	Inoculated plantlets	0.017	0.089	0.016	0.037	0.159	0.084	0.168	0.149	0.611	1.012
	Un-inoculated plantlets	0.009	0.13	0.015	0.046	0.2	0.109	0.162	0.115	0.629	1.014
	Conventionally-grown plants	0.009	0.083	0.015	0.04	0.147	0.083	0.139	0.102	0.533	0.857
	CD (*P*=0.05)	0.001	0.007	ns	0.002	0.019	0.006	0.006	0.004	0.01	0.026
	CV (%)	5.47	5.91	7.13	3.65	9.92	3.22	3.22	3.02	1.38	2.3
K	Inoculated plantlets	0.095	0.487	0.089	0.193	0.864	0.476	1.195	0.743	3.222	5.635
	Un-inoculated plantlets	0.03	0.824	0.067	0.175	1.096	0.385	1.029	0.506	2.305	4.224
	Conventionally-grown plants	0.049	0.237	0.087	0.209	0.582	0.433	0.329	0.511	2.089	3.428
	CD (*P*=0.05)	0.004	0.035	0.006	0.009	0.054	0.011	0.029	0.026	0.098	0.255
	CV (%)	5.38	5.76	6.34	4.07	5.41	2.24	2.87	3.87	3.32	4.94

Yield attributes, yield, quality and planting material of sugarcane

Inoculation of *G. dizotropicus* clearly increased the number of millable canes (8.15/clump) as compared to untreated micropropagated plantlets and control (Table 4). Micropropagated plantlets produced longer but thinner canes as compared to control, however, inoculation with endophyte showed marginal improvement in the cane girth (1.70 cm). The highest individual cane weight (0.610 kg) was recorded under conventional system. However, the reduction in average cane weight under micropropagated plantlets was compensated by number of millable canes produced and cane length finally resulting into higher cane yield under *in vitro* treated micropropagated plantlets (2.910 kg/clump). The improvements in yield attributes and yield of micropropagated plantlets might be due to stimulatory effects of hormones, vitamins, amino acids, micro and macro nutrients in the balanced medium used for plant growth and development. The increase in number of millable canes, cane length, number of internodes and finally the yield under endophyte treated micropropagated plantlets over un-treated micropropagated plantlets was due to higher biological nitrogen fixation through the microbial consortia in the rhizosphere as evident in Table 4. However, brix value remained at par among the treatments. Besides, the treated plantlets, produced 5.45 and 1.52 times more planting material (3 budded setts) over conventionally grown plants and un-treated micropropagated plantlets, respectively. Thus, 5.45 times more area can be planted using seed canes produced through inoculated micropropagated plantlets. Improvement in soil nutrient balance and cane yield have been reported under organic based integrated nutrient use including inoculation with strain IS 100 of *G. diazotrophicus* in the sugarcane varieties of sub-tropical India (Suman *et al.* 2005). Oliveira *et al.* (2006) also reported the positive effects of inoculation on the BNF contribution and cane yield in the micropropagated plantlets of Brazilian sugarcane variety SP70 1143 grown without nitrogen fertilization for three consecutive crops. Thus, BNF contribution was reckoned equivalent to the annual nitrogen fertilization.

Table 4 Growth, yield and quality parameters of sugarcane under three treatments

Treatment	NMCs/ clump	Cane length (m)	Cane diameter (cm)	No. of nodes/ cane	Single cane weight (kg)	Cane yield/ clump (kg)	Brix (%)	No. of three budded setts/ clump
Inoculated plantlets	8.15	1.95	1.70	23.9	0.357	2.910	19.06	64.9
Un- inoculated plantlets	6.68	1.73	1.30	19.2	0.315	2.104	18.98	42.8
Conventionally-grown plants	2.98	1.17	2.28	12.0	0.610	1.818	19.53	11.9
SEd	0.20	0.03	0.06	0.77	0.02	0.06	0.09	1.29
CD (*P*=0.05)	0.43	0.08	0.13	1.68	0.05	0.13	0.19	2.81
CV (%)	3.82	8.03	9.75	6.98	11.01	5.92	1.61	9.28

Thus, *in vitro* insertion of nitrogen-fixing bacteria (*Gluconacetobacter diazotrophicus* isolate IS 100) in micropropagated plantlets of sugarcane not only exhibits its role in breeder seed production but also diminishes the competition between the inoculated microbe and the native community during the initial stage of root colonization. The rapid multiplication of seed cane of newly released sugarcane varieties is very important for their faster adoption and resulting in the increase in the total sugarcane production. Micropropagation technology is being used for faster, healthy and disease-free planting material in seed production programme in sugarcane. Further, *in vitro*-treated micropropagated plantlets with *G. diazotrophicus* producing higher NMCs, increased cane length with higher number of nodes resulting into production of high rate of planting material (three budded setts) over other treatments in the present study. Conclusively, the inoculation of *Gluconacetobacter diazotrophicus* during development of micropropagated plantlets may be utilized for faster multiplication of seed cane of newly released varieties of sugarcane for their faster adoption in large area, which increases sugarcane production in sub-tropical India.

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