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Effect of Consortium of Nitrogen Fixing Endophytic Bacteria on Sucrose Metabolism and Nitrate Assimilation in Sugarcane (*Saccharum officinarum*)

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A B S T R A C T

Sugarcane is cultivated throughout the Indo-Gangetic plains of South Asia and Maharashtra is second largest producer. The leaf samples were collected at different stages of crop growth from Rahuri farm and were evaluated for nitrate assimilating enzyme viz., *in vitro* nitrate reductase and sucrose metabolizing enzymes activities viz., sucrose synthase, sucrose phosphate synthase and acid invertase. The experiment was laid out in R.B.D. with four replications and six treatments. Nitrogen fertigation was given as 100 % N through urea (T2), 50 % N + *Acetobacter diazotrophicus* (T3), 25 % N + consortium endophytic bacteria (T4), 0 % N + consortium endophytic bacteria (T5), 0 % N +without consortium endophytic bacteria (T6) and absolute control (T1). The results of the experiment revealed that enzymes activities viz., in vitro nitrate reductase, sucrose synthase, sucrose phosphate synthase and acid invertase by sugarcane crop was significantly higher in T4 treatment (25 % N + foliar application of consortium of endophytic bacteria) followed by Recommended Dose of Fertilizer (R.D.F.) treatment and 0% N with foliar application of consortium of endophytic bacteria. Hence, the use of foliar spray of consortium of N fixers @ 25 % concentration can save 75 % of nitrogen without affecting yield.

Introduction

Sugarcane is one of the important crop of Maharashtra and India. India is the second position in area, production and productivity in the world next to Brazil. India's contribution to the world is about 19%. In 2015-16, area in India was 4.927 Mha; production 348.48 million tones and productivity 70720 kg/ha (Anonymous, 2015).Sugarcane is a very exhaustive and extracting crop that removes about 205 kg N, 55 kg P_2O_5, 275 kg K_2O, 30 kg S, 3.5 kg Fe, 1.2 kg Mn, 0.6 kg Zn and 0.2 kg Cu from the soil for a cane yield of 100 t ha^{-1}. Consequently, due to both the nature of this crop and extensive cropping, the soils of the Indo-Gangetic plains are becoming nutrient deficient. In order to sustain productivity, major nutrients N, P and K are replenished each year at the recommended application rates, which in the sub-tropical part of India are 150 kg N ha^{-1} for the sugarcane plant crop and 220 kg N ha^{-1} for its ratoon crop as well

as 60 kg P_2O_5 and K_2O ha^{-1} for both the plant and ratoon crops. However, the efficiency of sugarcane to utilize applied N ranges between 16 and 45% as large quantities of applied N leach down through the soil layers due to the amount of irrigation required by the sugarcane crop (Suman *et al.*, 2005).

In addition, the continuous use of chemical fertilizers is causing an apparent deficiency in other micronutrients. The yields of sugarcane crops have plateaued and factor productivity has declined, with a decrease in soil organic matter status and deterioration in the physico-chemical and biological properties of the soil considered to be the prime reasons for the declining yield and factor productivity (Garside *et al.*, 1997).

The application of organic matter from such resources as animal manures, crop residues and green manuring has been shown to replenish organic carbon and improve soil structure and fertility (Guisquiani *et al*., 1995).

Moreover, several kinds of microbial agents capable of fixing N or mobilizing P and other nutrients are becoming an integral component of Integrated Nutrient Management System of crops. *Gluconacetobacter diazotrophicus* (earlier known as *Acetobacter diazotrophicus*), a N-fixing bacteria associated with sugarcane as an endophyte, is present in high numbers (as high as 106 counts g^{-1} plant tissue) in the root, shoot and leaves (Cavalcante and Dobereiner, 1988).

The exact role of such endophytic colonization, either individually or in a complex endophytic community, has not yet been elucidated, but the few inoculation experiments that have been carried out on micro-propagated plants suggest that positive colonization contributes to plant growth and development in terms of improved plant height, nitrogenase activity, leaf N, biomass and yield. Field trials conducted in India have shown that inoculation of *G. diazotrophicus* together with other diazotrophs or vascular arbuscular mycorrhiza (VAM) can match yield levels equal to the application of 275 kg N ha^{-1} (James *et al*., 1994; Sevilla *et al.*, 2001).

In Brazil, Baldani *et al.*, (1986) have reviewed the successful application of sugarcane N fixation in sugarcane breeding programs involving both local and introduced materials. In none of these programs where large amounts of N fertilizer utilized and because of this, their best materials have little demand for N fertilizers and an effective association has developed between endophytic N-fixing bacteria and the plant. Apart from N fixation, other properties associated with *G. diazotrophicus* are P-solubilization, the production of the plant growth hormone indole acetic acid (IAA) and the suppression of red rot disease (Suman *et al*., 2001).

Suman *et al*., (2005) reported that the native occurrence of *G. diazotrophicus* in sugarcane varieties of sub-tropical India is very low and that through the inoculation of efficient indigenous isolates, their number, plant N uptake and nutrient use efficiency could be increased at different N levels. Sugarcane has been found to respond positively to organic sources to meet its nutrient requirements; however, the effect of organic sources of nutrients together with *G. diazotrophicus* on crop yield and the availability and balance of nutrients in the soil along with biological and physical status and overall sustainability of the system need to be ascertained.

Hence, the present investigation was planned and carried out, to study the effect of consortium of nitrogen fixing endophytic bacteria on sucrose metabolism and nitrate assimilation in Sugarcane.

Materials and Methods

Treatment details

T_1 - Absolute control
T_2 - RDF (100% N, 75% P_2O_5, 100% K_2O and 25 t ha^{-1} FYM)
T_3- 50% N + *Acetobacter diazotrophicus* @10 kg ha^{-1}(Sett treatment)
T_4 - 25% N + Consortium of endophytic bacteria @ 3 L ha^{-1}[Foliar spray at 60 DOP]
T_5 - 0% N + Consortium of endophytic bacteria @ 3 L ha^{-1}[Foliar spray at 60 DOP]
T_6 - 0% N without consortium of endophytic bacteria

Extraction of enzyme

The fourth leaf of sugarcane collected at 90, 180, 270 and 360 Days After Planting (DAP) from P.G.I. Farm MPKV, Rahuri. Collected leaf samples were chopped into small pieces and representative sample extracted in a minimum volume of extraction buffer containing 100 mMTris. HCl, 2 mM EDTA, 2 mM, DTT, 10% glycerol and 2mM PMSF and centrifuged at 15,000 rpm for 10 min. Known volume of enzyme extraction collected in tube were tested for activity of enzymes *viz*., sucrose phosphate synthase (SPS) and sucrose synthase (SuSy), soluble acid invertase, *in vitro* nitrate reductase and nitrate.

Nitrate assay (Carole and Scarigelli, 1971)

The 20 – 100 mg of oven dried ground plant material is used for extraction and equivalent amount of activated charcoal were added to 100 ml conical flask having 20 ml of double distilled water. Boil the content for 3 – 4 minute. The extract then filtered through Whatman No. 1 filter paper; the residue was re-extracted and made up to suitable volume with glass double distilled water. Finally known concentration of nitrate 0.1 ml of sample was taken in 30 ml capacity culture tube and nitrate content was estimated compared with standard curve.

In vitro nitrate reductase assay (Hageman and Huckleshy, 1971)

The reaction was initiated by adding 0.5 ml NADH solution as the last component. In control tube NADH omitted instantly 0.5 ml distilled water was added. The tubes were incubated in water bath maintained at 30 ^{0}C for 30 min. Reaction was terminated by adding 0.2 ml of one molar zinc acetate solution which precipitate the protein, followed by 1.8 ml of 75 % ethanol. The precipitated was removed by centrifugation at 2,000 rpm for 5 minutes at room temperature and the supernatant was decanted in another test tube.

Nitrite formed by the reduction of nitrate was then estimated in suitable aliquot of supernatant by adding one ml each of sulphanilamide and NEDD solution respectively. The tubes were incubated at room temperature for 20 minute for colour development. Each of these test tubes, 7 ml of distilled water was added and colour mixed thoroughly on vertex mixer. The colour intensity was read on spectronic – 20 at 540 nm against the reagent blank. The amount was calculated from standard curve of nitrite and the *in vitro* nitrate reductase activity was expressed as µmoles of NO_2^- formed per mg protein per minute.

Sucrose synthase (SuSy) and Sucrose phosphate synthase (SPS) assay (Hawker, 1967)

The reaction mixture for sucrose synthase contained 125 µl 0.015 M UDPG, 125 µl 0.05 M fructose, 700 µl 0.2 M Tris-HCl buffer (pH 8.2) containing 0.025 M $MgSO_4$ and 50 µl of enzyme preparation in total volume of 1.0 ml. The reaction mixture for sucrose phosphate

synthase contained 125 µl 0.015 M UDPG, 125 µl 0.05M fructose–6-phosphate, 700 µl 0.2 M Tris- HCl buffer (pH 7.4) containing 0.025 M $MgSO_4$ and 0.4 M NaF (as phosphatase inhibitor) and 50 µl enzyme preparation in a total volume of 1.0 ml.

Sucrose was determined as per the method of Roe (1934) The reaction mixture of both sucrose phosphate synthase and sucrose synthase were incubated at 37^0C for 30 min and subsequently the tubes were kept in boiling water bath for 10 min and cooled. After cooling the tubes, 0.5ml 6% KOH was added and again kept in boiling water bath for 20 min. The cooled test extract was then used for sucrose estimation. To suitable aliquots of the test extract, 1ml resorcinol solution and 3 ml 75% H_2SO_4 were added and then incubated at 80^oC for 10 min. The intensity of pink colour was measured at 490nm and expressed the enzyme activity as µmoles of sucrose formed mg^{-1} protein min^{-1} and the concentration of sucrose was calculated from the standard curve prepared by using sucrose standard (10-100 µg ml^{-1}).

Soluble acid invertase assay (Vattuone *et al.*, 1981)

Leaf sample were collected 90, 180, 270 and 360 DAP and crushed by using liquid nitrogen and extracted in minimum volume of 50 mM sodium phosphate buffer (pH 7.5) containing 1mM β-mercaoptoethanol and 5 µM $MnS0_4$.The homogenate was centrifuged at 10,000 x g for 10 min. Soluble acid invertase activity was assayed by adding 50 µl enzyme to 750 µl of 50 mM sodium acetate buffer (pH 5.5). The enzyme reaction was started by addition of 0.2 ml 0.5 M sucrose solution and the reaction was terminated after 30 min by adding 1ml of alkaline copper reagent and kept the mixture exactly for 20 min. in boiling water bath. The tubes were cooled under running tap water or using ice and the reducing sugar produced was assayed by the method of Nelson (1944). The activity of the enzyme was expressed as µmole glucose formed mg^{-1} protein min^{-1}.

Soluble proteins

The soluble protein content of the enzyme extract was estimated as per the method described by Lowry *et al.* (1951).

Results and Discussion

Invitro nitrate reductase activity

The *in vitro* nitrate reductase (NR) activity in leaves of CoM-265 sugarcane cultivar planted at MPKV, Rahuri location analyzed at 90, 180, 270 and 360 DAP is depicted in Table 1. The mean *in vitro* NR activity was 184, 390, 564, and 184 µmoles of NO_2^- formed mg^{-1} protein min^{-1} at 90, 180, 270, 360 DAP, respectively.

The range *in vitro* NR activity was 137-225, 261-511, 422-689 and 138-255 µmoles of NO_2^- formed mg^{-1} protein min^{-1} at 90, 180, 270 and 360 DAP. The leaf NR activity was significantly higher in T_2 (RDF 100 % N) at all the growth stages followed T_4 treatment (25 % N + foliar application of consortium of endophytic bacteria) as compared to control. *In vitro* NR activity was higher.LI Dong-mei (2006) reported that effects of different NPK rates and ratios on enzyme activities in leaves of cucumber (*Curcumas sativa* L.). The results showed that an increasing the rate and ratio of nitrogen increase NR activity in leaf significantly.

Sucrose synthase activity

The sucrose synthase activity in leaves of CoM-265 sugarcane cultivar planted at MPKV, Rahuri location analyzed at 90, 180, 270 and 360 DAP was depicted in Table 2.

The mean sucrose synthase activity was 100.15, 48.11, 29.88 and 27.21 nmoles of sucrose formed mg^{1} protein min^{-1} at 90, 180, 270, and 360 DAP respectively. The range of sucrose synthase activity at Rahuri location was 70.5-155.8, 40.2-53.6, 26.2-33.7 and 23.4-33.7 nmoles of sucrose formed mg^{-1} protein min^{-1} at 90, 180, 270, and 360 DAP respectively. The maximum sucrose synthase activity was observed at T_4treatment (25% N + foliar application of consortium of endophytic bacteria) as compared to T_1(Absolute control) and T_2(RDF 100% N) treatment.

Sucrose phosphate synthase activity

The sucrose phosphate synthase activity of leaves in CoM-265 sugarcane cultivar planted at MPKV, Rahuri location analyzed at 90, 180, 270 and 360 DAP is depicted in Table 3. The mean sucrose phosphate synthase activity was 27.51, 23.1, 23.38 and 49.83 nmoles of sucrose formed mg^{-1} protein min^{-1} at 90, 180, 270, and 360 DAP respectively. The range of sucrose phosphate synthase activity was 16.5-40.3, 19-26, 20.9-26.5 and 42-59.2 nmoles of sucrose formed mg^{-1} protein min^{-1} at 90, 180, 270, and 360 DAP respectively. The maximum sucrose phosphate synthase activity was observed at T_4treatment (25% N + foliar application of consortium of endophytic bacteria) as compared to T_1(Absolute control) and T_2(RDF 100% N) treatment at 90, 180 and 270 DAP. The T_5 (0%N + foliar application consortium of endophytic bacteria) showed maximum activity at 360 DAP.

Table.1 Leaf *in vitro* nitrate reductase activity at various growth stages as influenced by consortium of endophytic bacteria in sugarcane at MPKV location

Treatment	*In vitro* nitrate reductase activity (nmoles of NO_2^- formed mg^{-1} protein min^{-1})			
	Days after planting (DAP)			
	90	180	270	360
T_1- Absolute control	142	261	422	142
T_2- RDF (100% N)	225	511	689	255
T_3- 50% N + *Acetobacter diazotrophicus* (sett treatment)	185	437	606	186
T_4- 25% N + foliar applicationof consortium of endophytic bacteria	222	474	659	222
T_5- 0% N + foliar Application of consortium of endophytic bacteria	163	378	533	163
T_6- 0% N without consortium of endophytic bacteria	137	278	472	138
Mean	184	390	564	184
Range	137-225	261-511	422-689	138-255
S.E. ±	3.85	10.66	7.32	3.85
C.D. @ 5%	11.60	32.15	22.07	11.60

Table.2 Leaf sucrose synthase activity at various growth stages as influenced by consortium endophytic bacteria in sugarcane at MPKV location

Treatment	Sucrose synthase (nmoles of sucrose formed mg^{-1} protein min^{-1})			
	Days after planting (DAP)			
	90	180	270	360
T_1- Absolute control	70.5	47.3	28.9	23.4
T_2- RDF (100% N)	138.7	53.6	28.5	33.7
T_3- 50% N + *Acetobacter diazotrophicus* (sett treatment)	105.2	43.4	32.2	24.3
T_4- 25% N + foliar Application of consortium of endophytic bacteria	155.8	53.5	33.7	30.6
T_5- 0% N + foliar Application of consortium of endophytic bacteria	70.6	50.7	26.2	26.2
T_6- 0% N without consortium of endophytic bacteria	60.1	40.2	29.8	25.1
Mean	100.15	48.11	29.88	27.21
Range	70.5-155.8	40.2-53.6	26.2-33.7	23.4-33.7
S.E. ±	0.017	0.005	0.0028	0.0016
C.D. @ 5%	0.051	0.016	0.0086	0.0048

Table.3 Leaf sucrose phosphate synthase activity at various growth stages as influenced by consortium of endophytic bacteria in sugarcane at MPKV location

Treatment	Sucrose phosphate synthase (nmoles of sucrose formed mg^{-1} protein min^{-1})			
	Days after planting (DAP)			
	90	180	270	360
T_1- Absolute control	21.2	19	25.6	49
T_2- RDF (100% N)	27.4	25.3	20.9	59.2
T_3- 50% N + *Acetobacter diazotrophicus* (sett treatment)	22	23.7	25	42
T_4- 25% N + foliar Application of consortium of endophytic bacteria	40.3	25.5	26.5	43.1
T_5- 0% N + foliar Application of consortium of endophytic bacteria	37.7	26	21.4	59.3
T_6- 0% N without consortium of endophytic bacteria	16.5	19.1	21.2	46.4
Mean	27.51	23.1	23.38	49.83
Range	16.5-40.3	19-26	20.9-26.5	42-59.2
S.E. ±	0.0028	0.0017	0.0023	0.003
C.D. @ 5%	0.0085	0.0053	0.0071	0.006

Table.4 Leaf soluble acid invertase activity at various growth stages as influenced by consortium of endophytic bacteria in sugarcane at MPKV location

Treatment	Soluble acid invertase (µmoles of glucose formed mg^{-1} protein min^{-1})			
	Days after planting (DAP)			
	90	180	270	360
T_1- Absolute control	0.133	0.061	0.040	0.006
T_2- RDF (100% N)	0.150	0.061	0.019	0.016
T_3- 50% N + *Acetobacter diazotrophicus* (sett treatment)	0.233	0.080	0.027	0.010
T_4- 25% N + foliar Application of consortium of endophytic bacteria	0.120	0.066	0.017	0.011
T_5- 0% N + foliar Application of consortium of endophytic bacteria	0.210	0.070	0.031	0.025
T_6- 0% N without consortium of endophytic bacteria	0.230	0.090	0.032	0.023
Mean	0.180	0.070	0.030	0.020
Range	0.120-0.233	0.0610.090	0.0190.040	0.0060.025
S.E. ±	0.01	0.005	0.002	0.001
C.D. @ 5%	0.04	0.01	0.008	0.003

LI Dong-mei (2006) reported that effects of different NPK rates and ratios on enzyme activities in leaves of cucumber (*Curcumas sativa* L.). The results showed that SS and SPS activities first increased and then decrease.

Acid invertase

The acid invertase activity in leaves of CoM-265 sugarcane cultivar planted at MPKV, Rahuri location analyzed at 90, 180, 270 and 360 DAP is presented in Table 4. The mean acid invertase activity was 0.180, 0.070, 0.030 and 0.020 µmoles of glucose formed min^{-1} mg^{-1}protein at 90, 180, 270, 360 DAP respectively. The range of acid invertase activity at Rahuri location was 0.120-0.230, 0.061-0.090, 0.019-0.040 and 0.006-0.025 µmoles of glucose formed mg^{-1} protein min^{-1} at 90, 180, 270, 360 DAP. The acid invertase activity was decreased at T_4treatment (25% N + foliar application of consortium of endophytic bacteria) as compared to T_1(Absolute control) and T_2(RDF 100% N). Lontom *et al.,* (2008) reported that, the activity of acid invertase was highest in the young internodes of sugarcane and it decreased with internodal age.

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References

Anonymous 2015. https://www.Indiastat.com

Carole, R.S., and Scaringelli, F., P. 1971.

Colorimetric determination of nitrate after hydrazine reduction to nitrite. *Microchemical J*. 16 (4): 657-672.

Cavalcante, V.A., and Dobereiner, J., 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant Soil*. 108:23-31.

Garside, A.L., Berthelson, J. E. and Richards, C. L. 1997. Effect of fallow history on cane and sugar yield of a following plant cane crop. *Proc. Sugarcane Tech*.19: 80–86.

Guisquiani, P.L., Paghai, M., Gighoth, G., Businelli, D. and Benetti, A. 1995. Urban waste compost: effects on physical, chemical, and biochemical soil properties. *J. Environ. Qual*. 24:75-182.

Hageman, R.H. and Hucklesby, D.P., 1971. *In vivo* nitrate reductase: *Methods in Enzymol*. 23:491-493.

Hawker, J.S., 1967. The activity of uridine–diphosphate glucose-Dfructose-6-phosphate 2-glucosyl transferase in leaves. *Biochem. J*. 105: 943-946.

James, E.K., Reis, V. M., Olivares, F., Baldani, J. I., and Dobereiner, J. 1994. Infection of sugarcane by the nitrogen fixing bacterium *Acetobacter diazotrophicus*. *J. Exp. Bot*. 45:757-766.

LI Dong-mei, W.Z., 2006. Effects of NPK rates and ratios on activities of metabolism enzymes in leaves of cucumber in greenhouse [J].*Journal of Plant Nutrition and Fertilizer* 12(3): 382-387.

Lontom, W., Kosittrakun, M. and Lingle, S. E. 2008. Relationship of acid invertase activities to sugar content in sugarcane internodes during ripening and after harvest. *Thai. J*. Lowry, O.H., Rose brough, N.J., Farr, A.l. and Randall, R.J. 1951. Protein measurements with the Folin Phenol Reagent. *J.Biol. Chem. Agril. Sci*. 41(3-4): 143-151.

Sevilla, M., Burris, R., H., Gunapala, N. and Kennedy, C. 2001. Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif mutant strains. *Mol. PlantMicro. Intera*. 14 (3): 358–366.

Suman, A., Gaur, A., Shrivastava, A. K. and Yadav, R. L., 2005. Improving sugarcane growth and nutrient uptake by inoculating *Gluconacetobacter diazotrophicus. Plant Growth Regul*. 47:155–162.

Suman, A., Shasany, A. K., Singh, M., Shahi, H. N., Gaur, A. and Khanuja, S. P. S. 2001. Molecular assessment of diversity among endophytic diazotrophs isolated from subtropical Indian Sugarcane. *World J. Microbiol. Biotechnol*. 17: 39-45.

Vattuone, M.A., Prado, F. E. and Sampietro, A. R. 1981.Cell wall invertases from sugarcane. *Phytochem*. 20: 189-191.

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