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ROOT ASSOCIATED NITROGEN FIXATION BY SUGAR CANE (*SACCHARUM OFFICINARUM* L. VAR. COL-54) IN PAKISTAN

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Abstract

Nitrogenase activity associated with roots of sugar cane (var. COL 54) was estimated from June, 1983 – April, 1984. Maximum mean acetylene reducing activity (ARA) was observed in preincubated unwashed roots (11-631 nmoles C_2H_4 g^{-1} dry roots h^{-1}) while washed roots (11-257 nmoles, C_2H_4 g^{-1} dry roots h^{-1}) and surface-sterilized roots (5-24 nmoles, C_2H_4 $root^{-1}$ h^{-1}) showed much lower activities. Nitrogenase activity was even observed in the roots which were not preincubated thus showing association of diazotrophs with the roots of sugar cane. ARA values were higher during October-December, 1983. Bacterial identification revealed that *Azotobacter chroococcum* was predominant in the rhizosphere of sugar cane.

Introduction

In many parts of the tropics, sugar cane (*Saccharum officinarum*) has been grown for centuries without addition of nitrogen fertilizers. Moreover this crop is poor in responses to N-fertilization (Ruschel, 1981). Association of N_2-fixing microorganisms with sugar cane roots was first demonstrated by Dobereiner (1959). Microbiology of this association was examined by Dobereiner (1961) who found higher proportion of *Beijerinckia* sp. in rhizosphere soil than in soil between rows. This was subsequently confirmed by acetylene reduction technique (Dobereiner *et al.*, 1972). Further studies on biological nitrogen fixation (BNF) associated with sugar cane were carried out in Egypt (Hegazi *et al.*, 1979), India (Jadhav & Andhale, 1976), South Africa (Purchase, 1980) and in Brazil (Ruschel, 1981) by using acetylene reduction technique (ARA).

The recent studies using ^{15}N showed direct evidence for dinitrogen fixation in sugar cane roots presumably originating from microorganisms inhabiting the root and also confirming translocation of the fixed nitrogen to the plant tissue when grown in solution culture (Ruschel *et al.*, 1981) and in the field (Matsui *et al.*, 1981). In Pakistan sugar cane is grown on 896,000 ha (Anon., 1985). Present investigations were carried out to look for any association and nitrogenase activity between sugar cane roots and N_2 fixing microorganisms in Pakistan.

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Materials and Methods

Sample collection: Samples of sugar cane (*Saccharum officinarum* var. COL-54) were collected from a selected field in Nuclear Institute for Agriculture and Biology (NIAB) during June, 1983 – April, 1984. Plant material alongwith large soil cores of 30 cm diam., and upto 21 cm depth were collected. Samples were analysed for nitrogenase activity on the same day. The soil adhering to the roots was gently removed and analysed for physico-chemical characteristics (Table 1).

Acetylene reduction assay (ARA): Acetylene reduction assays on excised roots of sugar cane (Dobereiner & Day, 1976) were done every two months for a period of one year. For the unwashed root assay, the soil adhering to the roots was gently removed. A portion of healthy roots was cut aseptically into small pieces (15-30 mm) and transferred to 30 ml screw capped bottle fitted with a silicone septum (220-400 mg^{-1} fresh root wt., $bottle^{-1}$). The gas phase was replaced by evacuation and flushing with nitrogen gas. This process was repeated for at least 6 times. Ten percent air was injected into the bottle and left overnight in an incubator at 30°C. Samples were then evacuated and refilled with N_2 gas. Acetylene (12% v/v) and air (1% v/v) were introduced by replacing the same volumes of nitrogen. All bottles were incubated at 30°C for 18 h in dark. Gas samples (100 μl) were analysed on a gas chromatograph (Carlo-Erba Model 180) fitted with a 1m x 2mm steel column filled with Porapak N (80-100 mesh) and a H_2 flame ionization detector (FID). Nitrogen was used as a carrier gas at a flow rate of 30 ml min^{-1}. Two controls, one bottle with C_2H_2 but without roots and other with roots but without addition of acetylene were also included during each assay.

For the washed root assay, a portion of roots was thoroughly washed with tap water until the supernatant liquid was clear of soil particles. Roots were finally washed with sterilized distilled H_2O and small root pieces transferred to 30 ml serum-capped bottles. For surface-sterilized root assay, a portion of washed roots was dipped in 0.1% $HgCl_2$

Table 1. Physico-chemical characteristis of composite samples of rhizosphere soil of sugar cane obtained at the time of analysis for nitrogenase activity.

Characteristics	June '83	Aug.	Oct.	Dec.	Feb.'84	April
pH (sp)	7.7	7.7	7.8	7.9	7.8	7.8
Saturation %	37.0	46.0	38.0	40.0	36.0	38.0
EC_e ($mS\ cm^{-1}$)	2.2	3.2	2.8	3.8	2.9	3.0
Total-N ($\mu g\ N\ g^{-1}$ soil)	7.4	7.3	7.4	7.1	7.1	6.3
NH_4-N (ppm)	5.6	3.5	7.0	3.5	2.8	2.8
NO_3-N (ppm)	0.0	1.4	9.8	2.8	2.8	3.5

for 30 sec. and washed repeatedly with sterile distilled water. Acetylene reduction assay was performed as described. ARA of excised roots without preincubation (Van Berkum & Sloger, 1979) was carried out. C_2H_2 was given to the freshly excised roots after flushing with N_2-gas. For each type of excised root assay, six portions of roots were obtained from three plants of sugar cane.

Bacterial identification: The most predominant N_2-fixing bacteria were isolated from the segments of roots exhibiting a high nitrogenase activity. Combined carbon (CC) solid medium of Rennie (1981) was used for the isolation of diazotrophs. All individual colonies were picked from CC plates and transferred to separate vials containing 5 ml of CC semi-solid medium. After 24 h of incubation at 30°C, the vials were sealed with a suba seal and the C_2H_2 reduction assay was performed. Each isolate exhibiting C_2H_2 reduction was streaked on nutrient agar (NA) to check its purity. Putative N_2-fixing (C_2H_2-reducing) bacterial isolates were identified on NA according to the scheme described by Claus (1979).

Results and Discussion

The nitrogenase activity of excised roots of sugar cane is presented in Table 2. ARA values are represented by mean as well as by ranges because a large variability between plants has been reported using this method (Van Berkum & Day, 1980). Frequency of samples exhibiting nitrogenase activity was always found to be higher (44-66%) in unwashed roots while it was low in the surface-sterilized roots (0.33%). The frequency of roots exhibiting nitrogenase activity varies considerably due to the absence of any specialized structure (= nodule) in this type of association. The nitrogenase activity in all the treatments was low. Unwashed roots exhibited higher nitrogenase activity (10-2415 nmoles C_2H_4 g^{-1} dry roots h^{-1}) while activity of surface sterilized roots was in the range of 1-56 nmoles C_2H_4 g^{-1} dry roots h^{-1}. The ARA values of unwashed roots obtained in the present analysis were higher than the figures reported earlier by Purchase (1980) from South Africa and by Rennie *et al.*, (1982) from Brazil. Usually higher nitrogenase activities were observed in washed roots. Protection of O_2 (Abrantes *et al.*, 1975) and production of organic acids and thus proliferation of diazotrophs (Van Berkum & Bohlool, 1980) are the usual explanations of such observation. In the present study, washed roots exhibited lower activity (1-528 nmoles C_2H_4 g^{-1} dry roots h^{-1}) indicating that most of the bacteria were present or atleast active on the exterior of the roots. Washing and surface sterilization of roots significantly reduced or completely removed C_2H_2 reducing isolates from the sugar cane plants and thus resulted in reduced associated C_2H_2 reducing activity. Patriquin *et al* (1980) indicated that N_2 fixing bacteria in the setts moved into the roots and colonized the rhizosphere of sugar cane after sprouting. Our study revealed that association of N_2-fixing bacteria with the roots of sugar cane was not tied under the local prevailing conditions as washing and subsequently surface sterilization of roots resulted in reduction or complete inhibition of acetylene reducing activity.

Table 2. Nitrogenase activity of excised sugar cane roots.

Months	Preincubated: Unwashed Frequency %	Unwashed Activity n mol C_2H_4 g^{-1} dry roots h^{-1}	Washed Frequency %	Washed Activity n mol C_2H_4 g^{-1} dry roots h^{-1}	Surface sterilized Frequency %	Surface sterilized Activity n mol C_2H_4 g^{-1} dry roots h^{-1}
June '83	44	20 ± 10.1 (10-50)	44	11 ± 3.8 (4-22)	22	7 ± 3.9 (5-18)
August	66	45 ± 16.4 (15-27)	46	25 ± 13.7 (7-65)	0	– –
October	56	183 ± 17.6 (70-642)	44	137 ± 67.5 (37-322)	22	24 ± 16.02 (8-56)
December	66	631 ± 365.9 (75-2415)	33	257 ± 38.1 (77-528)	22	17 ± 13.6 (5-139)
February '84	44	33 ± 18.8 (23-88)	44	5 ± 2.2 (1-11)	33	12 ± 15.6 (1-19)
April	66	11 ± 9.0 (16-72)	33	19 ± 11.7 (6-42)	11	5 ± 3.0 (2-8)

Months	Without preincubation Activity: Frequency	n mol C_2H_4 g^{-1} dry roots h^{-1}
June '83	56	19 ± 7.3 (3-43)
August	40	36 ± 18.0 (12-89)
October	56	41 ± 33.3 (12-274)
December	77	249 ± 59.2 (86-407)
February	56	27 ± 5.7 (2-39)
April	45	17 ± 13.2 (11-56)

Readings are mean of ARA positive samples.
± Standard error.
Extreme values are within brackets.

Table 3. Characteristics of different isolates from the rhizosphere of sugar cane.

Tests	Characteristics of isolates					
	Su1[a]	Su2	Su3	Su4	Su5	Su6
Gram reaction	–	–	–	–	–	–
Cellular morphology	Thin long rods	Thick rods	Long or short rods	Long or short rods	Thick rods	Thin rods
Motility	+	+	+	+	±	+
Colony morphology (-N medium)						
Az. ch. med[b].	+	Gummy	Gummy brown	Gummy brown	Gummy brown	Gummy brown
Az. vin. med[c].	–	Week growth	+	+	+	–
Na. benzoate med.	–	Week growth	+	+	+	–
Colony morphology (nutrient agar).	Spreading white	Entire flat	Convex entire	Convex entire	Convex entire	Convex entire
Pigment	–	–	Brown	Brown	Brown	Brown
Endospores	–	–	–	–	–	–
Oxidase	±	+	±	–	–	–
Catalase	+	+	+	+	+	+
$NO_3^- \rightarrow NO_2^-$	–	+	+	+	+	+
$NO_3^- \rightarrow N_2$	–	–	–	–	–	–
Urease	+	+	+	+	+	+
Hydrolysis of starch	+	+	+w	+	+	+
Indole	–	–	–	–	–	–
V.P.	+	+	+	+	+	+
Gelatin liquification	+	–	–	–	–	+
Citrate utilization	–	+	+	+	+	+
H_2S production	–	–	–	–	–	–
Acidification of glucose, sucrose & Mannitol	+	+	+	+	+	+
Rhamanose	+	–	+	+	+	+
B-galactosidase	+	–	–	+	+	+

a = Most occurring isolate of each successive samplings.

b = Medium for the growth of *Azotobacter chroococcum.*

c = Medium for the growth of *A. vinelandii.*

–, Negative, w, week; +, positive.

Su1 & Su2 related to *Beijerinckia* spp. while characteristics of Su3 to Su6 agrees well with *A. chroococcum.*

Nitrogenase activity associated with roots of sugar cane was seasonally dependent being maximum during the October-December (Table 2). In the present study nitrogenase activity associated with sugar cane was monitored for one year. Earlier studies were mostly performed at only one stage (Purchase, 1980; Rennie *et al.*, 1982).

Higher levels of combined N usually results in the suppression of root associated N_2-fixing activity (Van Berkum & Bohlool, 1980). Therefore, soil analysis for the total-N as well as of available N (NH_4^+ & NO_3^-) was carried out at each sampling time (Table 1). Analysis revealed the presence of meager amounts of available-N as well as of total-N at each stage and thus unable to interfere in the root associated N_2-fixation process.

Excised root assay with preincubation has been criticised by some workers (Lethbridge *et al.*, 1982; Van Berkum & Sloger, 1985). The ARA of excised roots of sugar cane was therefore measured without preincubation (Table 2). The frequency of positive samples in this assay was also higher in October-December, 1983. Activity was in the range of 2-407 nmoles C_2H_4 g^{-1} dry roots h^{-1}

In order to explore the possibilities of using N_2-fixing associations in the cultivation of economically important crops like sugar cane at reduced N fertilizer levels, a basic knowledge about the participating bacteria themselves as well as their behaviour, together with plants in aseptic and controlled systems is needed (Albrecht *et al.*, 1981). The most prevalent N_2-fixing bacteria at each successive samplings were identified using conventional tests. Additionally, commercial identification kits (API-20 and AP-50 CH, API-System France) were also employed as suggested by Rennie (1980) which covers 75 biochemical tests. Diagnostic characteristics are presented in Table 3. The C_2H_2 reducing isolates were dominated by family Azotobacteraceae. Majority of the isolates were related to *Azotobacter chroococcum* and *Beijerinckia* spp. Dobereiner (1961) found *Beijerinckia* sp in 95% of the rhizosphere soil samples of sugar cane plants from six Brazilian states. Ruschel *et al* (1978) isolated and identified *Azotobacter, Beijerinckia, Caulobacter, Clostridium, Derxia,* and a polar-flagellated vibrio. Hegazi *et al* (1979) could not find any *Beijerinckia* spp. associated with sugar cane roots in Egypt but *A. vinelandii, Klebsiella* sp., *Bacillus* spp. and *Spirillum* spp. were abundant. Purchase (1980) on the basis of cell morphology identified *Azospirillum* like bacteria from the roots of sugar cane in South Africa that exhibited C_2H_2 reduction. Rennie *et al* (1982) isoalted *Derxia, Enterobacter* and *Klebsiella* from the setts and roots of sugar cane growing in Brazil. In our study *A. chroococcum* was found to be most abundant. From this study it was not possible to estimate the frequency of occurrence of these bacterial strains in the rhizosphere of sugar cane. A more detailed study covering various sugar cane growing areas is needed to utilize this association for less dependence on fertilizers for the N-supply to this crop. Studies on quantification of the specific microorganism(s) in the presence of similar N_2-fixing bacteria in the soil and near the roots of sugar cane are also required.

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