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Development of indigenous nitrogen fixing bio-fertilizer for fertilizer saving and increasing productivity of sugarcane crop

Narayan Prasad Verma and Tapas Chowdhury

Abstract
Sugarcane is highly nitrogen requiring crop, as it requires about 250 kg of elemental nitrogen to grow successfully over a hectare of land. Cropping with the use of only inorganic fertilizers has led to depletion of biological fertility of the soil and posing threat to long term soil productivity. To stop the continuous decline in biological soil fertility it is important to use Bio-fertilizers in combination with chemical fertilizers. At present no suitable bio-fertilizer for sugarcane crop is available in Chhattisgarh state of India which is made up of indigenous strains of N-fixing bacteria. In this present study 110 bacterial isolates were isolated directly from field grown sugarcane plants and screened out 46 isolates on the basis of their N-fixing capacity *in-vitro*. Out of 46, 39 isolates were found capable to fix considerable amount of nitrogen (3.52 to 24.41 mg N/g of carbon). Highest quantity of nitrogen (24.41 mg) was fixed by isolate K-74 in semi solid agar medium. On the basis of colony characters and nitrogen fixation behavior the above 39 isolates were initially considered as Nitrogen fixing bacteria and four best performed isolates were further characterized morphologically by observing their growth on different media and physiologically by measuring growth at different temperature, pH, sucrose levels, and sensitivity towards antibiotics, IAA production ability and through other biochemical tests for confirmation. Further, these promising isolates were evaluated under field conditions to find out their efficacy on the growth performance and yield of sugarcane crop (Var. Co-085), compared with two national standard checks. Yield attributing characteristics of sugarcane crop *viz*. plant height, number of tillers, cane girth, number of nodes, brix% and yield were found to be significantly increased by isolate K-74 with 75% RDN. Maximum population density of the tested organism in soil (7.12 x10^7 cfu/gm), leaf tissue (4.4 x10^7 cfu/gm) and cane juice (20.2 x10^7 cfu/ml) was attributed to set inoculation of isolate K-74 with application of 75% RDN. Even it was found significantly superior over 75% RDN. It is concluded from the study that inoculation with indigenous isolate of K-74 can save more than 25% elemental nitrogen in sugarcane cultivation and could be an ideal bio-fertilizer for sugarcane in Chhattisgarh and other sugarcane growing states of India.

Keywords: Biological nitrogen fixation, sugarcane

1. Introduction
Sugarcane (*Saccharum officinarum*) is a sub-tropical crop which is the main source of sugar in India and holds a prominent position as a cash crop. India ranks first in production of sugarcane. It was occupied 50.42 lakh hectares during years 2017-18. In Chhattisgarh state of India, area under this crop was 0.433 lakh hectares during year 2018-19. In Chhattisgarh four sugar mills are established in Kabirdham, Balod and Surajpur districts. With the establishment of these factories, there has been an increase of area and production of sugarcane in the state and the growth was about 7-8% over precious years. Department of Agriculture, Chhattisgarh said that Chhattisgarh has traditionally been the productive state of paddy. Now the farmers are turning towards oilseeds, pulses and sugarcane. Sugarcane is highly nitrogen requiring crop. About 250 kg Nitrogen ha^{-1} is required for its successful cultivation. Looking to the high cost of chemical fertilizers the poor and marginal farmers of the state are unable to provide recommended dose of chemical fertilizers to the crop, resulting poor productivity of sugarcane. Other demerits of chemical nitrogenous fertilizer are the environmental problems like nitrate pollution in water bodies and increasing concentration of nitrous oxide and ammonia in environment due to long term fertilizer application which also lead to Global warming. In addition, chemical fertilizers can drain into ground water, streams, and rivers, risking human and animal health. So, initiative has been taken to find out the possibility of using alternative ways to reduce the use of chemical fertilizers in sugarcane cultivation.

At present no suitable bio-fertilizer for the sugarcane production is available in Chhattisgarh. Certain bacteria are capable to fix atmospheric nitrogen to soil & crop, *Gluconacetobacter* is one of them. It is capable of promoting plant growth through different mechanisms, such as biological nitrogen fixation (BNF), phytohormone production, phosphate solubilization and siderophore production (Pedraza, 2008) [21]. Biological nitrogen fixation by diazotrophs plays an important part in nitrogen cycling and attracted attention to the fact that they are one of the biggest natural sources of nitrogen inputs to soils and plants (Wartiainen *et al.*, 2008) [30]. Recently *Acetobacter diazotrophicus* is re-nomenclatured as *Gluconacetobacter diazotrophicus.* It is an acid loving bacterium; require pH 4.0 to 4.5 for growth and nitrogen fixation (Miyamoto *et al.*, 2008) [13]. *Gluconacetobacter diazotrophicus* has been described as potential diazotroph in Sugarcane. It is a gram negative, microaerobic nitrogen-fixing microorganism. In addition to nitrogen fixation it also secretes several organic acids which are helpful for crop growth. It has the potential to fix up to 150 kg ha^{-1} of atmospheric nitrogen. Associations of this endophytic, microaerobic, nitrogen fixing diazotroph with several crop species of economic importance and their response on field crops have been well documented (Subba Rao *et al.*, 1986) [28]. Aim of this study was to develop location specific indigenous Nitrogen fixing biofertilizer to reduce the nitrogenous fertilizer requirement of sugarcane crop and simultaneously to increase its productivity.

2. Materials and Methods

The study comprising (i) Collection of sugarcane set samples from several farmers' fields of sugarcane dominated district of Chhattisgarh for Isolation, characterization and identification of sucrose loving endophytic bacteria (ii) First stage screening of N_2 fixation isolates and (iv) Conduction of field experiment at farmers' field in Kabirdham district of Chhattisgarh as second stage screening of promising Nitrogen fixing isolates to test their efficiency for improvement of crop growth parameters. The above studies were conducted during 2018-19 in order to develop indigenous Nitrogen fixing biofertilizer for lowering the input cost of production and to get better productivity and profitability in sugarcane (*Saccharum officinarum*) cultivation in Chhattisgarh. The details of the studies conducted are as follows:

2.1. Collection of sugarcane samples

Sugarcane roots were collected from different fields where sugarcane was grown as a main crop annually. One hundred ten representative sugarcane root samples were collected from different villages of two blocks of Kabirdham district of Chhattisgarh state for isolation of Nitrogen fixing Bactria.

2.2 Isolation

The sugarcane plants were uprooted and root portion was separated and washed with tap water several times. The roots were then surface sterilized with 5% of sodium hypochlorite ($NaOC_l$) followed by 2 times washing by sterilized water (Meenakshisundaram, M. and Santhaguru, K., 2010) [18]. After washing, outer layer of sugarcane roots was removed by sterilized knife and made small pieces of them. The pieces were crushed aseptically to obtain juice. One ml juice of respective sample was suspended in N-free semi solid broth medium (Cavalcante and Dobereiner, 1988) [33] and incubated at 30 °C for 6-7 days. Yellowish orange bacterial growth formed in the tubes was streaked on LGI solid plates (Merasenla, *et al.* 2016) [16]. The morphological characters of the colonies were referred with the characteristics of Gluconacetobacter as described in Bergey's Manual of Determinative Bacteriology (2005).

2.3 Testing of Nitrogen fixing capacity of collected isolates

The Nitrogen fixing isolates, isolated from different sugarcane samples were tested for their nitrogen fixing capacity. The test was conducted in a semi-solid LGIP medium. Amount of nitrogen fixed by Nitrogen fixing isolates was estimated by Microkjeldhal (Ahmed *et al.* 2016) [4].

The formula for N_2 estimation is

N_2 (mg/g) = ml of H_2SO_4 in the sample x Normality of H_2SO_4 x 14.01 / Weight of the sample (Carbon used in grams).

2.4 Field experiment

A field level screening was conducted to know the efficiency of different Nitrogen fixing isolates for nitrogen fixation in field condition. Four top performing Nitrogen fixing isolates were tested along with two checks in field. The top four isolates were selected by conducting first stage screening study. The field experiment was laid out in Randomized Block Design taking sugarcane as a test crop and Co-085 as a test variety testing 8 treatments replicated four times. The treatments were T_1 (75% RDN + Gaceto-K-16), T_2 (75% RDN + Gaceto-K-40), T_3 (75% RDN + Gaceto-K-49), T_4 (75% RDN + Gaceto-K-74) T_5 (75% RDN + Rahuri), T_6 (75% RDN+Root-18), T_7 (75% RDN Un-inoculated-I) and T_8 (100% RDN Un-inoculated-II).

Different Nitrogen fixing isolates and standard checks were applied in the form of different treatments along with 75% recommended dose of nitrogen. Two separate pure inorganic treatments were included contained 75% and 100% recommended dose of nitrogen for comparison and to estimate the nitrogen fixation potential of Nitrogen fixing isolates. The nitrogen, phosphorus and potassium were applied at recommended dose of i.e. 250:116:80 kg/ha. The sets of the sugarcane were dipped in culture slurry containing 200 gm of individual inoculum mixed with 10 liters of water. In this mixture 250 gm jaggary was added. The sets were dipped in respective culture slurry for 15 minutes then planted in desirable plots. The experimental soil was clay loam in texture, containing available N-144.00 kg/ha, available P-7.25 kg/ha, available K-331.63 kg/ha with pH-7.3 and EC 0.15 dsm^{-1}. The population of Nitrogen fixing isolates in soil was 9.45 x 10^{-4} per gram of soil. At the time of harvest can height, cane girth, number of tillers, number of nodes, cane yield and brix% were recorded.

2.5 Determination of available NPK in sugarcane rhizosphere soil

After harvesting of crop, sampling of soil was done from ten locations of the experimental field and one composite soil sample was prepared. The samples were analyzed for available N, P, and K contents in addition to its physicochemical properties.

2.6 Plant (leaf) NPK analysis

The third leaf samples from the top were extracted from each reapplication after 10 months of planting. The leaf samples were dried and powdered. The digestion of leaf samples was

done and used for the estimation of N, P and K. (Piper, 1996) [22].

2.7 Population of Nitrogen fixing in soil, leaf and juice of sugarcane plant

The population was enumerated using semisolid N free semisolid LGI medium used for Nitrogen fixing isolates following MPN technique (Mote *et al*. 2017) [19]. The population was expressed as CFU/g oven dry soil. Representative surface soil sample of the experimental site was collected up to 30 cm depth before planting and after harvest of the crop to know the population of *Gluconacetobacter* (Chavan, 2015) [7]. Soil samples were kept in polythene pouches which were properly tagged and kept in refrigerator and analyzed immediately. Population of *Gluconacetobacter* in leaf and sugarcane juice was done at harvest. Leaf was washed in running tap water and leaf was cut in small pieces (0.2-0.3 cm) and washed with sterile distilled water 2 times. After washing leaves were serially diluted by sterilized water and population was enumerated in GYC media. The *Gluconacetobacter* population in sugarcane juice was done by taking 1 ml fresh juice using serial dilution and pour plate technique (Anitha, 2002) [2].

2.8 Statistical analysis

The data on different parameter *viz*, growth, yield and sucrose % of sugarcane were tabulated and analyzed by the method for analysis of variance. The statistical analysis at the level of significance used in 'F' test was P = 0.05. Critical difference values were calculated as per the methods given by Panse and Sukhatme (1985) [34].

3. Results

3.1 Testing of nitrogen fixation capacity of Nitrogen fixing isolates

The current research was conducted particularly for searching of effective indigenous Nitrogen fixing strain for sugarcane. Initially 46 sugarcane associated bacteria were isolated from different sugarcane growing locations (Table 1). They were tested for nitrogen fixing capacity in N free- semisolid medium. Out of 46 bacterial isolates 39 were found capable to fix considerable amount of nitrogen (3.52 to 24.41 mg N/g of carbon).

Table 1: Nitrogen fixation capacity of top ten Nitrogen fixing isolates *in-vitro*

Nitrogen fixing isolates	mg Nitrogen /g of carbon
Top ten local isolates	
K-74	24.41
K-40	22.43
-K-16	21.77
K-49	20.79
K-51	18.23
K-3	15.36
K-57	14.93
K-33	14.43
K-34	13.59
K-41	13.59
Rahuri (National check) **	18.43
Root-18 (Local check) *	15.93
	3.52-13.43
	(Rest 39 local isolates)
C.D.	0.75
SE(m)	0.267

National Check (**), Local check (*)

3.2 Field Experiment

Four Nitrogen fixing isolates and two standard checks were evaluated in field for their effect on the growth performance of sugarcane (C0-085). All the isolates were applied to field with 75% recommended dose of nitrogen (250 kg/ha). Two fertilizer N doses (75% RDN and 100% RDN) were also put in trial alone as Control-I & Control-II. The treatments have shown significant effect on plant growth (Table 3). Highest plant height was recorded in treatment 75% RDN + -K-74 i.e. 46.16, 175.66, 278.00 and 315.00 cm at 45, 150, 245 and 295 days after planting, respectively. Minimum plant height of sugarcane was recorded in treatment of 75% RDN at 45, 150, 245 and 295 days after planting. The cane girth of sugarcane was significantly affected by Nitrogen fixing inoculation. The treatment of 75% RDN+ K-74 recorded highest cane girth of 12.60 cm followed by 75% RDN+ K-40 at 295 days after planting. The minimum can girth of sugarcane was recorded (8.10 cm) in the treatment 75% RDN. The treatment 75% RDN+ K-74 recorded highest no of tillers (8.00) followed by 75% RDN+ K-40 at 295 days after planting. The number of nodes was also affected by inoculation. Highest no of node (23.10) was counted in case of 75% RDN+ K-74 followed by 75% RDN+ K-40 at 295 days after planting. Yield of cane over control-I i.e. 75% RDN was increased from 64.96 to 73.49, 75.65, 76.61 and 81.34 t/ha due to inoculation of local Nitrogen fixing isolates K-49, K-16, K-40 and K-74, respectively. In this study the sugarcane yield increased significantly from 70.03 to 81.34 t/ha due to Nitrogen fixing inoculation. Maximum yield was associated with local isolate no. K-74 with 75% RDN which found significantly superior over control-II i.e. 100% recommended level of nitrogen. The brix % of sugarcane juice was significantly improved with the inoculation of indigenous isolates and application of nitrogen fertilizer. The treatment of 75% RDN + K-74 recorded highest brix% of sugarcane (22.00) at 295 days after planting, followed by 75% RDN + K-40, whereas, minimum brix% (18.10%) was associated with solo application of 75% RDN at 295 days after planting.

Table 2: Effect of Nitrogen fixing with different levels of fertilizer nitrogen on plant growth parameters and yield of sugarcane

	Treatment	Days after planting				No. of Tillers	Girth (cm)	No. of nodes	Yield (t/ha)	Brix %
		45	150	245	295					
T_1	K-16+75% RDN	41.66	165.87	271.95	305.65	7.4	10.82	20.5	75.65	21.46
T_2	K-40+75% RDN	43.74	169.54	275.92	307.36	7.5	11.33	21	76.61	21.1
T_3	K-49+75% RDN	39.66	164.09	271.21	301.16	7.2	9.6	19.9	73.49	20.3
T_4	K-74+75% RDN	46.16	175.66	278	315	8	12.6	23.1	81.34	22
T_5	Rahuri (National check) +75% RDN	38.12	160.28	263.89	290.97	6.5	8.7	17	72.62	20
T_6	Root-18 (Local check) +75% RDN	38.79	158.15	269.98	297.08	6.9	9.2	19	71.11	19.3
T_7	Control (75% RDN)	36	151.97	233.2	250.87	6.1	8.1	15.1	64.96	18.1
T_8	Control (100% RDN)	38.79	160.49	261.72	290.32	6.4	8.65	17.2	70.03	19
SE(m)		1.548	4.55	7.63	9.43	0.3	0.37	0.94	2.75	0.7
C.D.		4.63	13.39	22.45	28.31	0.94	1.13	2.87	8.35	2.09

RDN-recommended dose of nitrogen

3.3 Soil (residual) and leaf nitrogen content

The soil nitrogen content of sugarcane significantly increased due to the inoculation of indigenous isolates and application of fertilizer nitrogen. The treatment of 75% RDN + K-74 recorded highest nitrogen content in soil (169.60 kg/ha) at 295 days after planting, followed by 75% RDN + K-40 treatment, whereas, the minimum soil nitrogen content was recorded (138.00 kg/ha) in the treatment of 75% RDN at 295 days after planting. Similarly, the leaf mineral nitrogen content of sugarcane has shown significant enhancement due to the inoculation of indigenous isolates and application of fertilizer nitrogen. The treatment of 75% RDN+ K-74 recorded highest nitrogen content in leaf i.e. 3.45% at 295 days after planting, followed by 75% RDN+ K-40. The minimum leaf nitrogen content (2.52%) of sugarcane was associated with the treatment 75% RDN (fig. 1).

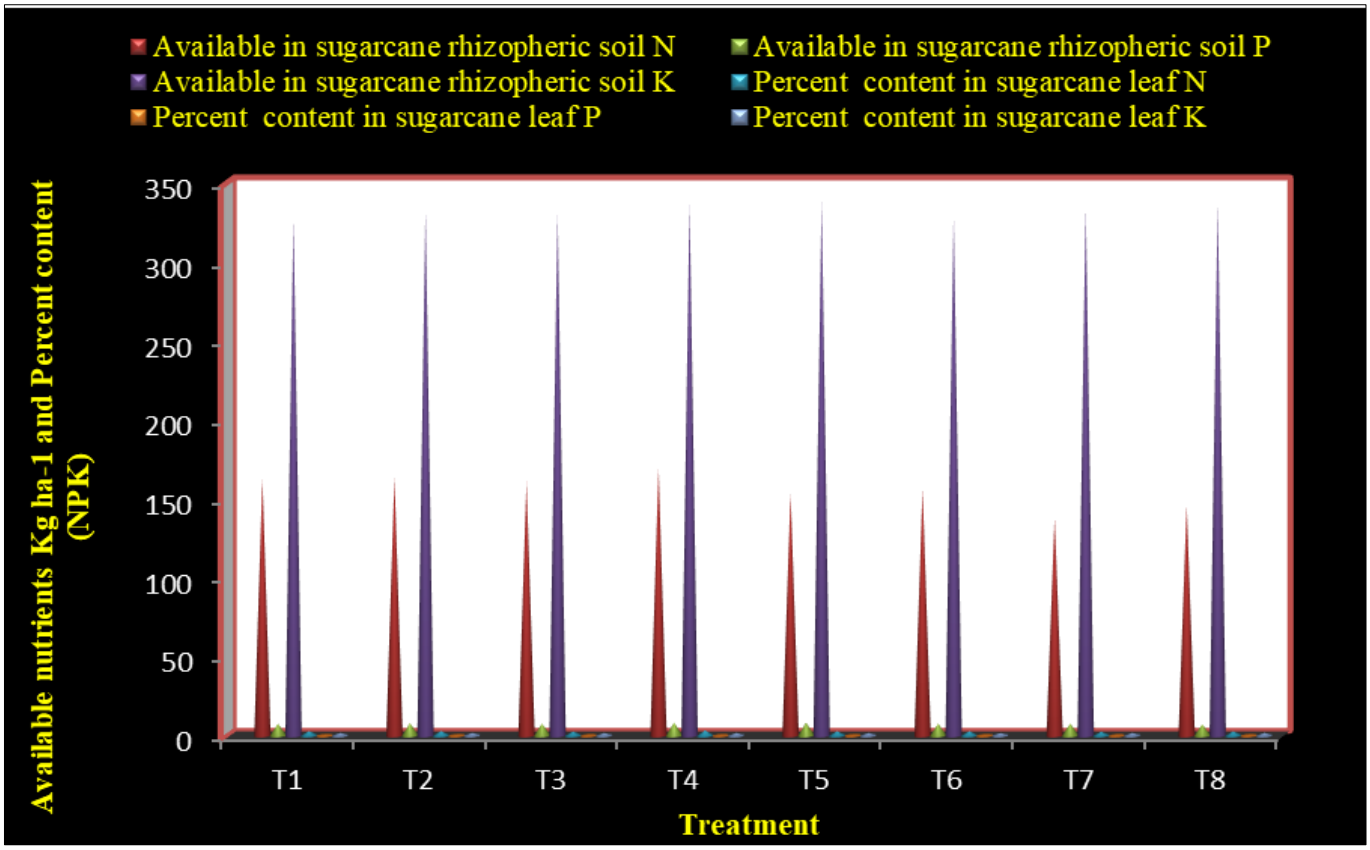

Fig 1: Available NPK in soil and Percent NPK content in leaf at 295 (DAP) with inoculated and un-inoculated treatments of Nitrogen fixing isolates

Treatments

T_1	K-16+75% RDN	T_3	K-49+75% RDN	T_5	Rahuri (National check) +75% RDN
T_2	K-40+75% RDN	T_4	-K-74+75% RDN	T_6	Root-18 (Local check) +75% RDN
T_7	Control (75% RDN)	T_8	Control (100% RDN)		

3.4 Population study of Nitrogen fixing isolates at harvest

The population data of Nitrogen fixing isolates (fig. 2) in sugarcane grown soil, leaf and juice was shown a variation due to the inoculation of Nitrogen fixing isolates and application of 75% level of fertilizer nitrogen (fig. 2). Population of Nitrogen fixing isolates was highest recorded in treatment T_4 (75% RDN+K-74) which was at par with T_7 (75% RDN) and T_8 (100% RDN).

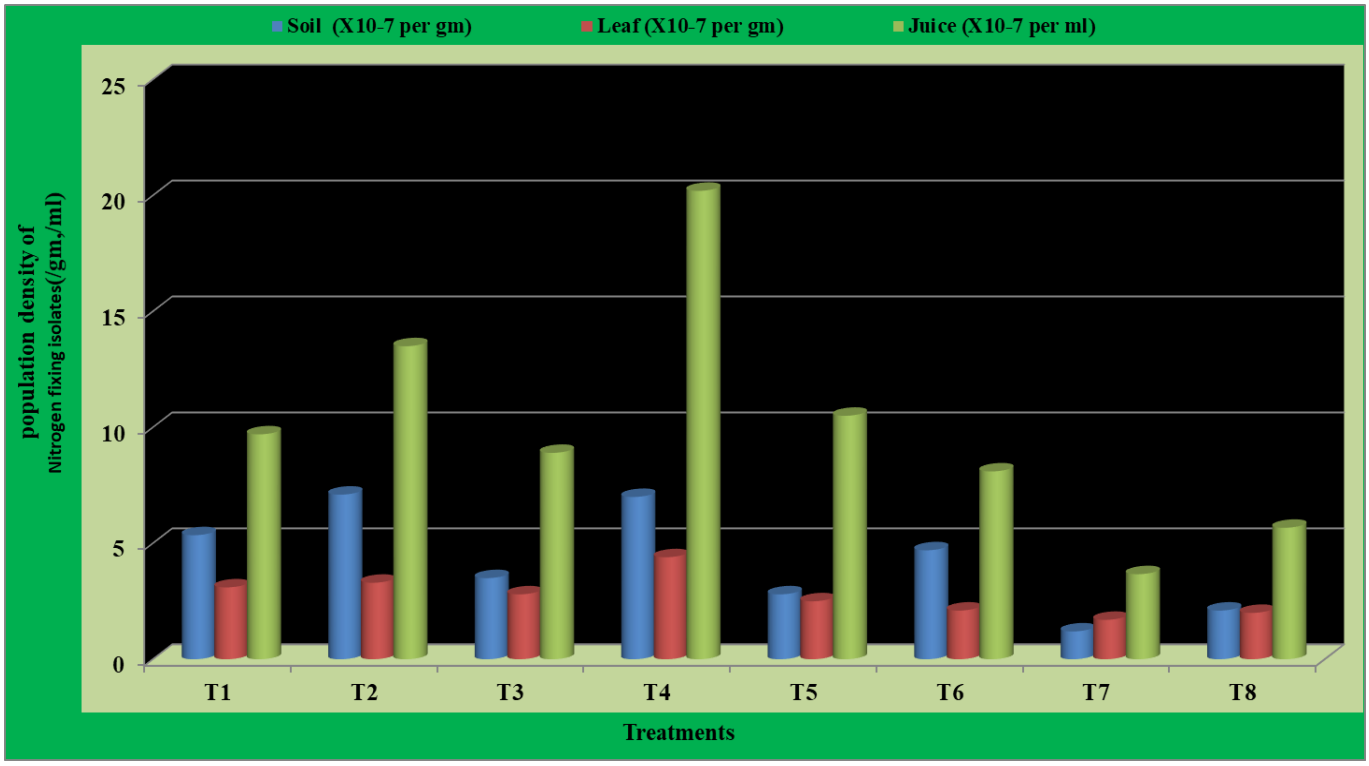

Fig 2: Population density of Nitrogen fixing isolates in soil ($X10^{-7}$/gm), leaf ($X10^{-7}$/gm) and juice ($X10^{-7}$/ml)

Treatments

T_1	K-16+75% RDN	T_3	K-49+75% RDN	T_5	Rahuri (National check) +75% RDN
T_2	K-40+75% RDN	T_4	K-74+75% RDN	T_6	Root-18 (Local check) +75% RDN
T_7	Control (75% RDN)	T_8	Control (100% RDN)		

4. Discussion

The present study showed the search of an efficient native strain of Nitrogen fixing suitable to grow, multiply, establish in soil and in the plant system to provide substantial amount of nitrogen to the host plant in its active stage of growth which in turn produce the maximum yield. Initially forty-six isolates of dizotrophic endophytic bacteria Nitrogen fixing were isolated from sugarcane roots in different district of Chhattisgarh state of India. Further, the nitrogen fixing ability of the collected isolates was evaluated by grow them in N-free semisolid medium. Out of 46 isolated, 39 were found efficient to fix considerable amount of nitrogen in growing medium. Hughest quantity of nitrogen was fixed by isolate K-74. This was supported by the findings of Hema *et al.* (2017) [10] who mentioned the nitrogen fixing capacities of *Gluconacetobacter* bacterium.

Under the present study, in the field experiment the plant growth parameters like height, can girth, number of tillers and nodes have clearly indicated the contribution of 25% nitrogen requirement of sugarcane crop. The above findings also supported by Krishnaswamy and Muthusamy (2010) [15], who mentioned use of 50% N along with *Gluconacetobacter* significantly increased girth of sugarcane plant even over 100% N. The improvement of crop performance due to inoculation of *Gluconacetobacter* may also be due to N supplementation along with other hormonal effects of bacteria as they produced several hormones identified under laboratory conditions (Muthukumarsamy *et al.*, 1999b) [17]. In yield study, the maximum yield of cane was associated with isolate no. Gaceto-K-74 which fertilized with 75% RDN. The yield was recorded significantly higher over control i.e. 100% RDN. Similar response towards yield increment was also reported by several researchers. Thangaraju and Govindrajan (2001) [29] conducted field trial in low soil N condition, reported that *G. diazotrophicus* increased the cane yield (114.67 ton/ha) with 75% RDN even over 100% RDN which produced 102.67 ton of cane per hectare. Past research work of Kolage *et al.*, (2001) [14] and Soomro *et al.*, (2013) [27] also envisaged that crop productivity of sugarcane increased due to *Gluconacetobacter* inoculation with low N application.

The residual soil nitrogen content in sugarcane grown soil increased significantly due to inoculation of indigenous isolates of *Gluconacetobacter*. The inoculation of Gaceto-K-74 and 75% fertilizer N application recorded highest to contribute 169.60 kg residual nitrogen. The soil N contribution by the bacterium was also confirmed by Chavan, (2015) [7] who found an increment of residual soil N from 230.08 to 225.06 kg/ha due to inoculation of *Gluconacetobacter* with 50% fertilizer N. Bioinoculation of nitrogen fixing bacteria with nutrient fertilizer might be complementary and supplementary to each other which supplied NPK regularly. The nitrogen content in sugarcane leaf was increased significantly and found maximum due to inoculation of *Gluconacetobacter* with 75% RDN observed at 295 days after planting. Rajkumar *et al.*, (2017) [23] observed an accumulation of 1.82% N, 0.65% P and 1.25% K in leaf tissue as an effect of consortium of endophytic nitrogen fixing bacteria. Study on population of *Gluconacetobacter* in soil, leaf and juice revealed that inoculation of *Gluconacetobacter* with 75% of fertilizer N significantly increased its population in plant body and in external soil environment. Caballero-Mellado *et al.*, (1995) also mentioned that at low level of

fertilizer N (50% RDN) with addition of *Gluconacetobacter* favoured its population. Thangaraju & Anitha, (2010) [1] observed the enhancement of population of *Gluconacetobacter* to the tone of $31x10^6$ after 5 months of inoculation on leaf compared over the population of 10^3 CFU/gm leaf observed at the time of planting at 50% RDN level.

5. Conclusion

This study indicated that inoculation of sugarcane crop by indigenous efficient *Gluconacetobacter* bacterium is beneficial for overall growth improvement and production of higher yield of sugarcane. There is no loss of yield of cane despite the reduction of 25% recommended amount of fertilizer N, so there is a vast possibility of sugarcane growers to reduce input cost during sugarcane cultivation by means of curtailing fertilizer N by adopting the *Gluconacetobacter* bacteria for cultivation under low input technology.

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