JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2012. Vol. **6**(3), p. 1351-1355

Diversity of Free Living Nitrogen Fixing Bacteria in Sugarcane Rhizosphere of Barak Valley, Assam

Debamita Chakraborty*, G.D. Sharma[1] and B. Deb[2]

[1]Department of Life Science, Assam University Silchar - 788 011, India.
[2]Department of Botany, G.C College, Silchar - 788 004, India.

(Received: 21 February 2012; accepted: 05 April 2012)

Three different types of nitrogen fixing bacteria *viz*, *Azotobacter vinelandii*, *Bacillus polymyxa* and *Pseudomonas fluorescens* were isolated from rhizosphere of field-grown sugarcane in Barak Valley, Assam. On an average highest number of diazotrophs was recorded in rainy season, which was followed by summer season. Lowest number of diazotrophs was observed in winter season. Nitrogenase activity of *Azotobacter vinelandii, Bacillus polymyxa* and *Pseudomonas fluorescence was* 403.05, 209.15, 107.80 (nM C_2H_2/h / mg protein) respectively. Data obtained in this work will hopefully contribute to future research aimed at developing reliable and effective sugarcane inoculants.

Key words: Diazotrophs, diversity, Sugarcane rhizosphere, nitrogenase activity.

Sugarcane is one of the main cash crops in Barak Valley, Assam. Its cultivation occupies about 4500 ha of land area in Barak Valley. Its two main products are sugar and alcohol, a clean renewable alternative fuel. The type of sugar produced by sugarcane is sucrose, which is the most important of all the sugars. Sucrose is used as a sweating agent for food and in the manufacture of cakes, candies, preservatives, soft drinks, alcohol, and numerous other foods. Thus, there is a great demand of sugarcane in the world's economy.

Nitrogen is one of the most essential nutrients for the growth of sugarcane. Soil of Barak Valley is poor in nitrogen. Use of chemical N fertilizer can increase sugarcane yield. But the constant use of chemical fertilizer on soil brings adverse effects on soil metabolic processes and ultimately renders the soil into a less unproductive mass. In view of the above facts, attention has been given to the use of biofertilizesr which on one hand increase fertility of the soil and on the other hand do not cause any harmful effects on soil[1]. Research on biological nitrogen fixation (BNF) has increased significantly because of its potential importance to the economy and the environment. It has been especially interesting and important to find BNF in nonlegumes like sugarcane, rice, kallar grass and maize[2,3,4]. Nitrogen-fixing bacteria of the genera *Azotobacter*, *Enterobacter*, *Bacillus*, *Klebsiella*, *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia* and *Azoarcus* have been reported in association with a wide range of grasses [5,6,7]. Nitrogen-fixing micro-organisms have been reported living in the rhizosphere and as endophytes of sugarcane cultivars. The most

* To whom all correspondence should be addressed.
Mob.: +91-9435079602;
E-mail: chakrabortydebamita09@gmail.com

common bacteria isolated from sugarcane tissues are *Gluconacetobacter diazotrophicus*, *Herbaspirillum rubrisubalbicans* and *H. seropedicae* [8,9]. Other micro-organisms such as *Enterobacter cloacae* and *Klebsiella oxytoca* have also been reported to be found inside sugarcane [10]. Application of biofertilizers provides equivalent output to 30-40 kg/ha N chemical fertilizer. Evidence for biological nitrogen fixation in sugarcane (*Saccharum spp*.) was reported in Brazilian sugarcane varieties. Studies on long-term N-balance and 15N isotope dilution technique [11] also have shown that some sugarcane varieties may actually obtain up to 70% of their N requirements by nitrogen fixation .The aim of the work is to study the diversity of free living nitrogen fixing bacteria in sugarcane rhizosphere of Barak Valley, Assam.

Materials and methodology

Collection of samples

Three sugarcane fields each from Cahar, Karimganj and Hailakandi districts were sampled during the study period in three different seasons *viz,* summer, rainy and winter. Samples from the rhizosphere soil, were collected, according to Muthukumarasamy *et al* (1999) [12] for the isolation of nitrogen fixing endophytes. Soils chemical and granulometric analyses were performed as described by Soil Conservation Service (1975) [13].

Isolation and culture of nitrogen fixing diazotrophs

Isolation and culture of nitrogen fixing diazotrophs was carried out by the dilution plate method of Waskman (1961) [14].10g of soil sample was added with 100ml of sterile distilled water in a conical flask and shaken on horizontal shaker to form a homogenous soil suspension. Serial dilution upto 10^{-6} was made.1 ml from 10^{-6} dilution was inoculated aseptically into three sterilized petridishes containing 20 ml of melted Burk's medium. Upon solidification, the *plates* were incubated in inverted position for three days at 25^0C.The total number of colony forming units of diazotrophs per g of soil was counted. Discreate well developed and separated selected bacterial colonies were subcultured from each Burk's agar plate. Single colonies were restreaked on Burk's agar plate for further purification.

Characterization of aerobic diazotrophs

Pure isolates of the aerobic nitrogen-fixing bacteria from rhizosphere were characterized using the criteria of Bergey's Manual of Systematic Bacteriology (1994)[15] and Bergey's Manual of Determinative Bacteriology (1984)[16] .The following morphological, physiological and biochemical tests were used: Colony morphology, size, Gram staining, production of diffusible and non-diffusible pigments were determined on Burk's solid medium after 2 and 5 days of incubation at 30 æ%C. Motility was determined in wet mounts and flagella arrangement assessed by the technique of Rhodes(1958)[17].Encystment was induced by the method of Socolofsky and Wyss (1961)[18]. The cyst was stained by the method of Vela and Wyss(1964) [19] .Poly-*â*-hydroxybutyric acid (PHB) granules were examined according to the method described by Baker(1967) [20].Utilization of glucose, rhamnose, caproate, caprylate, meso-inositol, mannitol and malonate as carbon source was assayed on Burk's basal medium with a final concentration of 0.5% (w/v) of each substance. Starch hydrolysis was tested in cultures on Burk's solid medium containing 1% (w/v) potato starch by flooding with Lugol's iodine. Growth at different pH values was assessed by absorbance measurements (540 nm) after 48 h incubation on liquid Burk's medium with the pH adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0.

Nitrogenase activity

Nitrogenase activity of the isolated diazotrophs was assessed by acetylene reduction test .Isolates from Burk's N-free media were grown, for 48 h at 30^0C. Each vial was sealed with rubber stopper and the head space (5 mL) was injected with 10% (v/v) acetylene. Gas samples (0.2 mL) were removed after 1 h and assayed for ethylene production with a gas chromatograph using a hydrogen flame ionisation detector. Ethylene contamination of the acetylene was always known and accounted for final calculations. Values were expressed as nmoles C_2H_2 h^{-1} mg^{-1}

RESULTS AND DISCUSSION

The average rainfall of 72 mm, 461 mm and 1090 mm was recorded during winter, summer and rainy season respectively. The average (maximum and minimum) air temperature ranged from 15 -23^0C in winter, 26-36^0C in summer and 24-30^0C in rainy season during the study period. Average relative humidity of 76.5%, 80.14% and

91% was recorded in winter; summer and rainy seasons respectively. Soil of the study site was found to be clay- loamy type with low organic carbon, nitrogen, phosphorus and potassium contents. Highest soil temperature was recorded in summer 25^{0}C, which was followed by rainy 23^{0}C and winter season 18^{0}C respectively, while highest soil pH was recorded in rainy seasons (5.62), which was followed by summer (5.61) and winter season (5.60) respectively. Highest moisture content was recorded in rainy season 74% followed by summer (44%) and winter season (40%).Three species of diazotrophs viz, *Azotobacter vinelandii, Bacillus polymyxa* and *Pseudomonas fluorescence* were isolated from sugarcane agroecosystem soil of Barak Valley, Assam. This study is supported by that of Graciolli *et al* (1983) [21] who found *Enterobacter cloacae*, *Bacillus polymyxa*, *Azotobacter vinelandii, Erwinia herbicala* and *Klebsiella pneumoniae* associated with root and rhizosphere of sugarcane. *Viswanathan et al* (2003) [22] also, isolated *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida* from sugarcane rhizosphere.

The data in the Table – (1) represented the population of diazotrophs in sugarcane rhizosphere of the three districts of Barak Valley, Assam in different seasons. On an average highest number of diazotrophs was recorded in rainy, season followed by summer season. Lowest number of diazotrophs was observed in winter season. Marshall and Devinny (1988)[23] also observed low microbial population during the winter due to low temperature and soil moisture content whereas, summer season supported large active populations.

The population of diazotrophs differed significantly between different seasons

Table 1. Quantitative estimation of soil diazorophs in sugarcane rhizosphere of three districts of Barak Valley, Assam

District	Diazotrophs	Number of cells/g of dry soil (x10^6)		
		Summer	Rainy	Winter
Cachar	*Azotobacter vinelandii*	20 ± 3.02	25 ± 1.32	0
	Bacillus polymyxa	17 ± 2.96	22 ± 2.42	12 ± 1.72
	Pseudomonas fluorescence	16 ± 2.40	21 ± 1.93	13 ± 2.82
Karimganj	*Azotobacter vinelandii*	0	12 ± 2.54	10 ± 3.25
	Bacillus polymyxa	6 ± 3.08	11 ± 1.39	9 ± 2.67
	Pseudomonas fluorescence	7 ± 4.01	10 ± 3.04	8 ± 3.28
Hailakandi	*Azotobacter vinelandii*	18 ± 1.56	23 ± 0.78	0
	Bacillus polymyxa	15 ± 2.38	20 ± 1.48	10 ± 4.02
	Pseudomonas fluorescence	14 ± 1.45	19 ± 0.89	11 ± 3.85

± =standard error, Number of cells/g of dry soil is the average of all the location in each three district

Table 2. Analysis of variance of the data on quantitative estimation of soil diazorophs in sugarcane rhizosphere of three districts of Barak Valley, Assam

District	Source of variation	Degree of freedom	Calculated value of F	Significance level
Cachar	Between seasons	*dft* = 2 *dfw* = 9	7.808	*
Karimganj	Between seasons	*dft* = 2 *dfw* = 9	6.44	*
Hailakandi	Between seasons	*dft* = 2 *dfw* = 9	9.42	*

*= significant at 5% probability level

(Table 2).The seasonal variation of diazotrophs population was highest in Hailakandi district.

The pure cultures of diazotrophs were tested for their ability to fix atmospheric nitrogen. The nitrogenase activity of *Azotobacter vinelandii* was found maximum (403.05 nM C_2H_2 hr^{-1} mg^{-1} protein), followed by *Bacillus polymyxa* (209.15 nM C_2H_2 hr^{-1} mg^{-1} protein) *and Pseudomonas fluorescenec* (107.80 nM C_2H_2 hr^{-1} mg^{-1} protein) respectively. This finding is supported by the study of Deb Roy *et al* (2009)[24].

CONCLUSION

Sugarcane harbors diverse population of endophytic, epiphytic and rhizospheric diazotrophic bacteria and the rhizospheric diazotrophs that have been isolated and identified from sugarcane growing fields of Barak Valley, Assam were only a relatively small fraction of this much larger community/population which needs further study to assess their contribution in the nitrogen cycle in sugarcane agroecosystem.

ACKNOWLEDGMENTS

Thanks are due to authorities of Assam University, Silchar and G.C Colleg , Silchar for providing /laboratory facilities to carry out the research work.

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