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ASSESSMENT OF NATIVE DIAZOTROPHIC STRAINS IN NITROGEN NUTRITION OF RICE *CV.* IR-36 IN SOUTH ASSAM

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Diazotrophic bacteria were isolated by soil dilution plate method from the low lying acidic rice growing fields of South Assam in sali (autumn) cropping season'2007 and the isolates were identified following Bergey's manual of determinative bacteriology. On screening the distribution of these diazotrophic isolates in the soil it was observed that the isolates viz., *Azotobacter chroococcum*, *Azospirillum amazonense* and *Beijerinckia indica* were with highest frequency and relative abundance. To assess the efficacy of these strains in N-nutrition in clayey low lying submerged acidic rice field soil of South Assam, slurry of charcoal based inoculants of the diazotrophs was prepared and rice (*Oryza sativa* L. *cv.* IR-36) seedlings were inoculated by seedling root dip method. At different growth stages of inoculated rice plants, analysis of growth and yield parameters and activity of nitrogen assimilatory enzymes were carried out from representative samples and compared to that of uninoculated rice plants. It was observed that the activity of nitrogen assimilatory enzymes and growth and yield of rice plants were increased significantly by all the diazotroph strains over un-inoculated control plants. On an average the glutamine synthetase (GS) activity was highest in *A. chroococcum* inoculated plants whereas glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activity was higher in *A. amazonense* inoculated plants indicating greater capacity of ammonia assimilation of the latter strain. The increase in the growth and yield parameters of inoculated plants over uninoculated control ranged from 17-49% with *A. chroococcum* and *A. amazonense* showing highest capacity of supplying N-nutrition to rice *cv.* IR-36. It is evident from the present investigation that *A. chroococcum*, *A. amazonense* and *B. indica* strains can be used as efficient biofertilizers for rice cultivation in Southern parts of Assam as the strains are predominant diazotrophs of rice agro-ecosystems.

Keywords: Biofertilizers; Diazotrophs; Glutamate dehydrogenase (GDH); Glutamate synthase (GOGAT); Glutamine synthetase (GS).

Introduction

Rice is the staple food of the people of South Assam and is grown in 80% of its cultivable land, but the rice productivity of the zone is much below (1350 kg/ha) the national average (2691 kg/ha)[1]. One of the probable causes of poor rice production may be due to low (<50%) agronomic use efficiency of nitrogenous fertilizers in acidic rice field soils under high rainfall (>2000 mm) conditions[2]. Therefore, most of the rice field soils of South Assam are deficient in N and the farmers are using excessive amount of chemical N fertilizers. Since the long term use of chemical nitrogenous fertilizers depletes the soil organic matter and poses a threat to the survival of indigenous soil micro-flora biological nitrogen fixation (BNF) technology can play an important role in substituting the use of chemical N fertilizers in rice cultivation. In South Assam rice crops are grown in both wetland and upland agro-ecosystems. However, about 90% of the total rice cropped area is under wetland cultivation. In upland system aerobic bacteria fix atmospheric N while in wetland both aerobic and anaerobic bacteria fix N. N_2-fixing diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis, improve nutrient uptake, enhance stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate[3]. Indirectly diazotrophs are able to decrease or prevent the deleterious effects of pathogenic microorganisms, mostly through the synthesis of antibiotics and /or fungicidal compounds, through competition for nutrients or by the induction of systemic resistance to pathogens[3]. In addition they can affect the plant indirectly by interacting with other beneficial microorganisms. This suggests that there is a potential role for plant growth promotive effect of diazotrophic bacteria if appropriate bacteria and growth conditions are conducive. Keeping in view the above facts, the present investigation was undertaken to assess the native diazotrophic bacterial strains associated with the rice agro-ecosystems of South Assam in N-nutrition of rice (*cv.* IR-36).

Material and Methods

South Assam is located between latitude 24⁰ to 25⁰ North and longitude 92⁰ to 93⁰ East. The zone is bounded by N. C. hills and Jaintia hills on North, Mizoram in the South, Manipur on East and Tripura and Bangladesh on West. The total geographical area of the zone is 6941.2 sq. km., 8.84% of the total area of the Assam state [4]. The zone has been divided into three districts namely Cachar, Karimganj and Hailakandi. The diazotrophs were isolated from the low lying acidic rice fields of the three districts of South Assam during sali (autumn) cropping season'2007 under submerged condition by dilution plate method of Waksman[5]. The Burk's Agar medium was used for isolation of diazotrophs. Identification of diazotrophs was done by micro-morphology, cultural, biochemical and physiological characters following Bergey's manual of determinative bacteriology[6]. Among the identified diazotrophs the most frequent and abundant strains were *Azotobacter chroococcum*, *Azospirillum amazonense* and *Beijerinckia indica*. Therefore, these strains were used as bioinoculant for field grown rice *cv.* IR-36 in sali (autumn) season'2008 (August to December). For this purpose a low lying rectangular rice field of size (12 X 10 sq. mt.) was selected at the outskirt of Silchar (Kalinjar). The field was divided into 12 micro-plots of uniform size (1 X 10 sq. mt.) to grow the rice crop following bioinoculation with the three strains and control in triplicate. The standard inoculums of the strains were prepared by growing the bacteria in 250 ml glucose-peptone broth for 72 hours at 28±2⁰C on a shaker. The cells in active growth stage were harvested by centrifugation at 8000 rpm for 10 minutes and resuspended the pellets in sterile distilled water to attain a concentration of 10^8 cfu ml^{-1}. Then sterilized charcoal powder was mixed with the aqueous suspension of the diazotroph strains to prepare carrier based inoculants. Plant infection of the strains was done by seedling root dip method. For this purpose aqueous slurry of charcoal powder based inoculants of the strains was made in shallow plastic tubs. Fifteen day old seedlings of rice were uprooted carefully and the root portions of the uprooted seedlings were dipped into the aqueous slurry for 12 hours to ensure maximum contact of diazotrophs on the root surface. The seedlings treated with aqueous slurry of sterilized charcoal powder but devoid of diazotrophs strains used as control. Three replicates were kept for each treatment.

The three strains of diazotroph were tested for their ability to fix atmospheric N_2 by assaying the activity of nitrogenase enzyme following acetylene reduction technique [7].The activity of nitrogen assimilatory enzymes was also assayed in the root fraction of the paddy plants at 30, 60 and 90 days after sowing. For enzyme assay, washed and sterilized 1g root material was extracted with 5 ml of 100 mM phosphate buffer (pH 7.5) containing 1mM disodium EDTA, 1mM dithioerythritol and 1% polyvinyl pyrrolidone (PVP) for glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). The mixtures were centrifuged at 12000 rpm for 30 minutes at 4⁰C in Beckman ultracentrifuge and the supernatant collected was used for the subsequent enzyme assay. 1g root material was extracted with 5 ml of 50mM imidazole chloride and 20% glycerol at 4⁰C. The mixture was centrifuged at 12000 rpm for 30 minutes and the supernatant collected was used to assay glutamine synthetase (GS) activity. GS was estimated by the method described by Shapiro and Stadtman[8], GOGAT and GDH were estimated by the method described by Vandecasteele *et al.*[9] and Doherty[10], respectively, using the enzyme extracts. At the harvesting stage, the plants were uprooted with intact roots, washed thoroughly to remove the adhered soil and taken for analysis of growth and yield parameters. Plant height, number of tillers /plant and weight of 100 grains were measured in standard scale. The N-content of leaves was estimated by micro-kjeldahl method [11] and the chlorophyll content of leaves was estimated by Arnon method[12]. Protein content of grains was determined by Lowry's *et al.*[13] method. The fresh plants were dried in hot air oven at 85⁰C for 36 hours for uniform drying and the dry biomass was measured in standard scale. Summary statistics was used to obtain the mean and percent increase over control[14]. The least significant difference was calculated following the method of Misra and Misra [15].

Results and Discussion

The diazotrophs were isolated on semisolid agar media from the low lying acidic rice field soils of South Assam and the results were presented in the Table 1-4. Among the isolated diazotrophs the most dominant strains were *Azotobacter chroococcum*, *Azospirillum amazonense* and *Beijerinckia indica* showing highest percentage of occurrence. These three strains are distributed covering a large area in rice agro-ecosystems of South Assam with higher number of viable cells. These strains were identified on the basis of their biological parameters (Table 1) and biochemical characteristics (Table 2). *A. chroococcum* strain was identified on the basis of smooth opaque convex circular gummy colony with undulated margin, cells ovoid

Table 1. Biological parameters of native diazotrophic strains isolated from the rice fields of South Assam.

Diazotroph strain	Colony characteristics	Cell shape	Cell size	Nature of growth	Flagella	Cyst
Azotobacter chroococcum	Smooth opaque convex circular gummy colony with undulate margin	Ovoid rods or cocci	2 X 1.5 µm	Aerobic	Peritrichous	Present
Azospirillum amazonense	Subsurface white pellicle in semi-solid medium	Curved rods	2 X 0.5 µm	Microaero-philic	Peritrichous	Absent
Beijerinckia indica	Smooth irregular folded and raised colony with tenacious and elastic slime	Straight rods with rounded ends	2 X 1 µm	Aerobic	Peritrichous	Present

Table 2. Biochemical characteristics of diazotrophic strains.

Diazotroph strain	Gram reaction	Catalase reaction	Carbon source utilization			Biotin require-ment	Polysac-charide granule/ gum production
			Glucose	Sucrose	Malate		
Azotobacter chroococcum	–	+	+	+	–	–	+
Azospirillum amazonense	–	Variable	+	–	+	–	+
Beijerinckia indica	–	+	+	+	–	–	+

Table 3. Effect of diazotroph inoculation on activity of nitrogen assimilatory enzymes in rice *cv.* IR-36.

Treatment	Glutamine synthetase (GS)[1]			Glutamate synthase (GOGAT)[2]			Glutamate dehydrogenase (GDH)[2]		
	30 d	60 d	90 d	30 d	60 d	90 d	30 d	60 d	90 d
Azotobacter chroococcum	222.4	387.9	174.8	172.4	220.5	69.2	141.3	182.7	61.4
Azospirillum amazonense	212.6	357.2	169.3	180.4	207.3	82.1	153.2	200.2	87.7
Beijerinckia indica	189.3	272.4	120.6	164.8	201.4	57.2	1176.1	173.7	69.4
Control	127.2	172.4	54.3	121.8	168.4	47.1	103.9	156.2	41.4
LSD (P<0.05)	40.43	100.93	63.07	27.40	42.97	30.13	37.30	35.15	31.87

1 - nM of glutamyl hydroxamate formed min^{-1} mg^{-1} of protein; 2 - nM of NADH oxidized min^{-1} mg^{-1} of protein. LSD (P<0.05) =Least significant difference at 5% level of significance.

rods or cocci, size 2 ×1.5 µm, older cells produce brown to black pigments, presence of thick walled cysts, presence of flagella, cells occur singly or in pairs or in chains or in irregular clumps, gram-negative, catalase positive, aerobic and produce extracellular gum. *A. amazonense* strain was identified on the basis of white dense pin point colony with irregular margin, subsurface white pellicle in semisolid medium, cells vibroid rods, size 2 × 0.5 µm, absence of cysts, gram negative, catalase variable, micro-aerophillic, presence of flagella and polysaccharide crystals in the cell. *B. indica* strain was confirmed on the basis of smooth irregular folded and raised colonies, produces tenacious and elastic slime, cells curved rods, size 2.0 × 1.0 µm, occur singly, presence of cysts, gram

Table 4. Effect of native strains of diazotroph on the growth and yield of rice *cv.* IR-36.

Treatment	Plant height (cm)	Dry biomass (g)	No. of tillers/ plant	Weight of 100 grains (g)	Chlorophyll a content of leaves (mg/g dry weight)	Nitrogen content of shoot (%)	Protein content of grains (%)
Azotobacter chroococcum	142.6 (129)	48.75 (149)	17.2 (128)	2.50 (120)	1.02 (131)	0.64s (142)	10.23 (131)
Azospirillum amazonense	136.1 (123)	44.05 (134)	17.3 (129)	2.66 (128)	1.08 (138)	0.59 (131)	9.73 (125)
Beijerinckia indica	138.2 (125)	38.25 (117)	16.2 (121)	2.58 (124)	0.92 (118)	0.56 (124)	9.15 (117)
Control	110.4 (100)	32.82 (100)	13.4 (100)	2.08 (100)	0.78 (100)	0.45 (100)	7.80 (100)
LSD (P<0.05)	20.70	13.45	3.03	0.56	0.32	0.17	0.77

*Each variety is the average of three replicates; Figures in bracket indicate percent increase over control; LSD (P<0.05) =Least significant difference at 5% level of significance.

negative, absence of flagella, catalase positive, aerobic and presence of intracellular polysaccharide crystals (Table 1,2).

The above strains were screened for their N_2-fixing potential by assaying the activity of nitrogenase enzyme following acetylene reduction technique. The maximum nitrogenase activity was revealed by *A. chroococcum* (413.15 C_2H_2 hr^{-1} mg^{-1} protein) followed by *A. amazonense* (393.13 C_2H_2 hr^{-1} mg^{-1} protein) and *B. indica* (300.30 C_2H_2 hr^{-1} mg^{-1} protein).

To test their efficacy in N_2-nutrition the strains were used as bioinoculant for growing rice *cv.* IR-36. Table 3 reveals that *A. chroococcum* treated plants has significantly higher GS activity followed by *A. amazonense* at all the growth stages. The GS activity decreased at the harvesting stage as compared to the initial stages of growth. The GDH activity was highest in *A. amazonense* treated plants followed by *A. chroococcum* and *B. indica* different growth stages. GDH activity also decreases towards the harvesting stage of the plant. With regard to GOGAT activity, inoculation with the *A. amazonense* strain showed higher activity at 30 and 90 days after transplantation. After 60 days of growth the higher GOGAT activity was showed by *A. chroococcum* inoculated plants (Table 3).

The data in Table 4 revealed that treatment with all the strains of diazotroph significantly increased the growth and yield of rice over untreated control plants. At the harvesting stage maximum plant height was reported in *A. chroococcum* treated plants. Almost 29% increase in plant height over uninoculated control was revealed by *A. chroococcum* inoculation. *A. amazonense* and *B. indica* treatment improved plant height by 23% and 25%, respectively. The highest dry biomass was shown by plants treated with *A. chroococcum* strain and 48% increase in dry biomass over control was reported. *A. amazonense* and *B. indica* have improved the dry biomass by 34% and 16%, respectively at the harvesting stage. Highest number of tillers was shown by *A. amazonense* inoculated plants and an increment of 21% to 29% over control was recorded. The maximum improvement in grain weight (28%) was seen in *A. amazonense* inoculated plants followed by *B. indica* (24%) and *A. chroococcum* (20%). The chlorophyll a content of leaves was also improved over control on inoculation with the diazotroph strains. Highest Chlorophyll a content was shown by *A. amazonense* treated plants followed by *A. chroococcum* and *B. indica.* 18% to 38% increase in the plant chlorophyll a content was reported at the harvesting stage. The nitrogen content of shoot was improved by 42%, 31% and 24% following inoculation with *A. chroococcum*, *A. amazonense*, and *B. indica,* respectively. The protein content of grains was also increased over the uninoculated plants. 31% increase in protein content of matured grains was observed in *A. chroococcum* inoculation. *A. amazonense* and *B. indica* increased protein content of grains by 25% and 17%, respectively (Table 4).

On screening the frequency and relative abundance of the diazotrophic isolates three dominant strains of diazotrophs viz., *A. chroococcum*, *A.*

amazonense and *B. indica* were selected to test their efficacy to improve the growth and yield of rice *cv.* IR-36 in absence of chemical N fertilizer. Occurrence of diazotrophs like *Azotobacter*, *Azospirillum* and *Beijerinckia* in the paddy fields of South Assam has similarity with the findings of Bhattacharjee *et al.*[16]. Persistence of diazotrophic bacteria such as *A. chroococcum*, *A. amazonense* and *B. indica* in the acidic rhizosphere soil of graminaceous plant like rice supported the earlier work of Narolia *et al.*[17]. In the present investigation occurrence of *A. amazonense* strains in the low-lying submerged acidic rice fields of South Assam is similar to the findings of Thakuria *et al.*[1]. Among the three strains the highest nitrogenase activity was shown by *A. chroococcum* isolate and the nitrogenase activity range of the strains was 114.80-413.15 nM C_2H_4 hr^{-1} mg^{-1} protein which is in conformity with the findings of Naureen *et al.*[18].

The rice field soil of South Assam is acidic due to heavy annual rainfall (>2500 mm) and the average soil pH ranged from 5.0 to 6.5. The strains of *A. chroococcum*, *A. amazonense* and *B. indica* were isolated from the acidic rice fields of South Assam and the strains are more or less acid tolerant. In tropical low lying acidic rice fields, the genera *Azotobacter*, *Azospirillum* and *Beijerinckia* were frequent, demonstrating a good capacity for their survival[19,20] and a widespread occurrence especially in the rhizosphere soil of rice[21]. Among the mechanisms used by these bacterial species to overcome unfavourable conditions are cyst formation, flocculation, melanin production, synthesis of poly-β-hydroxybutyrate (PHB), polysaccharide synthesis and association with mycorrhizal fungi[21].

The activity of nitrogen assimilatory enzymes in inoculated plants was higher than the uninoculated control. Fallik *et al.*[11] also reported that specific activities were higher in *A. brasilense* inoculated to maize plants and further reported that the enzyme activity increased from 2nd and 3rd week after sowing. After physiological maturity stages, *i.e.,* at the harvest stage, the enzyme activity decreased.

In the present investigation the GS activity was higher than GOGAT and GDH activity in plants inoculated with all the strains. The *A. chroococcum* inoculated plants showed highest specific activity of GS at initial growth stages. At the harvest stage the highest GS activity was found in *A. amazonense* inoculated plants. Higher activity of GA and GOGAT than GDH revealed that ammonia assimilation was carried out in the plant cytoplasm by the coupled enzyme complex system of the two enzyme: GS-GOGAT. Yuan *et al.*[22] reported that GS-GOGAT cycle appeared to be the only pathway for ammonia assimilation in the developing rice grain. Ohyama and Kumazava[23] also reported that ammonia was assimilated by GS-GOGAT system. Kush *et al.*[24] reported that assimilation of ammonia formed in the process of nitrogen fixation primarily by the reaction catalysed by glutamine synthetase. Since the total nitrogen content of shoot in *A. chroococcum* inoculated plants was highest compared to the other strains, the GS activity was higher accordingly. The lowest activity of nitrogen assimilatory enzymes was shown by *B. indica* inoculated plants and the specific activities were still higher than the uninoculated control. Among the three strains the highest GDH activity was revealed by *A. amazonense* inoculated plants and this may be due to higher capacity to utilize ammonia than the other strains. The GDH activity was lower in plants inoculated with all strains than GS and GOGAT activity at all stages of growth and this fact may be attributed to the presence of low ammonia concentration in the field as the field was left un-cropped in the previous cropping season.

Recently, studies are being carried out in different parts of the world about the role of diazotrophic inoculation in crop growth and yield to develop alternative sources of chemical fertilizers. *Azospirillum*, phosphate solubilizing bacteria (PSB) and fluorescein pseudomonads (FP) isolates increased yield by 27.8-100%, 6.13-21.6% and 28.3-54.7% over the yield of uninoculated control rice plots [25]. In the present investigation plant height, dry biomass, number of tillers/plant, weight of 100 grains, $chlorophyll_a$ content of leaves, nitrogen content of shoot and protein content of grains were increased by 23-29%, 17-49%, 21-29%, 20-28%, 18-38%, 24-42% and 17-31%, respectively over uninoculated control rice plots. The rhizosphere of rice harboured *Azotobacter* and *Beijerinckia* strains along with *Azospirillum* species even under submerged condition in low lying acidic rice field of Assam [26]. Total biomass yield, total chlorophyll content and plant height were also found to be increased with the inoculation of *Azotobacter* and *Azospirillum* applied with or without lime and FYM [27]. Reports showed that seed inoculation of *Azotobacter sp.* on rice have improved growth, nitrogen content, chlorophyll content, grain weight and yield of crops in absence of chemical nitrogen and phosphate fertilizers at its maximum level. The native strains of diazotrophs from the rice field soils were best suited to the ecophysiological condition of rice agro-ecosystems of Southern Assam. The above experiment supports our findings in the present investigation on the efficacy of diazotrophic inoculation to improve the growth and yield of rice *cv.* IR-36 grown in field condition in autumn (sali) season. Overall, it is evident that the acidic rice field soils of Southern parts of Assam harbour good number of *A. chroococcum*, *A. amazonense* and *B. indica* strain as the major diazotroph with higher frequency and relative abundance which have showed potential for N_2 fixation and can be developed as efficient strains of biofertilizers for rice.

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