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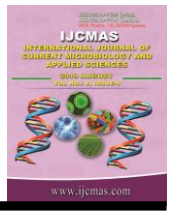

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Isolation and Characterization of Associative Diazotrophs from Lowland Rice Ecosystem

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A B S T R A C T

Rice crop harbours diverse beneficial plant growth promoting diazotrophs which mostly plays a significant role in supplementing not only N fertilizer but also gives indirect benefit to the crops by improving their productivity and immunity. Considering all the benefits from PGP diazotrophs, rice rhizospheric soil samples were collected randomly from 20 different locations of Jorhat district, Assam. Altogether, 53 rhizobacterial cultures were isolated in non-selective TSA media. Further, 30 pure culture isolates were grown in selective media to select diazotrophic PGPR out of which five phosphate solubilizers, three *Azospirillum*, one *Azotobacter* and one *Pseudomonas* for finding multiple PGP traits. Rhizobacterial cultures were morphologically and biochemically characterized and indicated that none of the culture were identical to each other. On qualitative evaluation, all selected cultures produced ammonia, IAA, HCN and urease activity but Siderophore and Antimicrobial activity were limited to few cultures. The test cultures produced nitrogenase activity ranged from 5 to 12.6 µmol C_2H_4 $ml^{-1}hr^{-1}$ and ammonia production from 112.34 to 310.05 mgl^{-1}. Diazotrophic PGP cultures P 20 (*Azospirillium*) was found the most efficient and superior culture among the test isolates followed by P 49 (PSB) and P 51 (PSB) in respect of PGP activities likes biofertilizers, phytostimulators and bio-control properties.

Introduction

The application of chemical fertilizers and pesticides achieved satisfactory results but at the same time it is threatening the agricultural processes, such as pollution of large water resources, destruction of ecosystem, increase susceptibility of the crop to diseases, acidity of the soil and reduction in soil fertility (Kumar and Satyanarayan, 2012). Extensive use of nitrogen fertilizer depletes the soil organic matter and poses a threat to the survival of heterogenous soil microflora. In such condition the use of bioinoculants comprising PGPR presently have emerged as a promising approach in augmenting plant growth and development by suppressing plant disease (Bioprotectant), improving nutrient acquisition (Biofertilizer) and stimulating phytohormone production (Biostimulants) which can be a

good alternative to chemical fertilizers and pesticides (Welbaum *et al.,* 2004) or can be used as component in Integrated Nutrient Management System with reduced rates of agrochemicals against broad spectrum plant pathogen (Saharan and Nehra, 2011).

Rice crop harbours diverse communities of beneficial microbes such bacteria, fungi, archeae, protozoa and algae. The rhizosphere is a thin layer of soil which immediately surrounds the plant roots and is the active sites for root activity and microbial proliferation (Gray and Smith, 2005). Naturally occurring soil bacteria that aggressively colonize plant roots and accelerate plant growth via direct and indirect mechanism are called Plant Growth Promoting Rhizobacteria (PGPR) (Kumar *et al.,* 2011).

Beneficial microbes that fix atmospheric nitrogen by reducing dinitrogen to ammonia or simply N_2 fixers are called 'diazotrophs' and during interaction with the crop, it helps the growth and development by improving productivity and immunity (Dilworth, 1974) via directly or indirectly. On inoculation to the crop, PGPR can directly synthesize phytohormones (IAA) or facilitate the uptake of nutrients (N_2-fixation, P-solubilization, K-mobilization and P- mobilization, micronutrient solubilization) and improve soil fertility and indirectly prevent some of the phytopathogenen through various mechanisms by producing siderophore, antibiotics, HCN for disease resistance, antimetabolite to avoid predation and competition, β-1, 3, glucanase, chitinase, (Laskar *et al.,* 2013). Some of the beneficial diazotrophs that act as PGPR in rice rhizosphere are: *Azoarcus, Acetobacter, Azorhizobium, Azospirillum, Bacillus, Burkholderia, Herbaspirillum, Klebsiella* and many other beneficial microbes. However, their activity and abundance depend on the soil environment (Islam *et al.,* 2009; Ritu *et al.,* 2017).

Considering the multiple benefit of diazotrophic PGPR, the present study was undertaken to isolate and characterize diazotrophic PGPR from rice rhizosphere and to assess their role in PGP activities and bioinoculant properties.

Materials and Methods

The present investigation was carried out during 2015-2017, in the department of Soil Science, Assam Agricultural University; Jorhat. Rice rhizospheric 5-10 cm soils in bulk along with the roots were collected randomly from 20 different lowland rice fields under Jorhat district of Assam and isolated the rhizobacteria through serial dilution spread plate technique (Vincent, 1970; Somasegaren and Hoben, 1994) using non selective TSA (Tryptic Soy Agar) media. Diazotrophic PGPR such as P-solubilizer was isolated using Pikovskaya's medium, *Azospirillum* on semi solid NFB (Nitrogen free bromothymol blue) medium (Dobereiner and Freitas, 1978), *Azotobacter* on Jensen medium and *Psuedomonas* on the King's B medium. Altogether 10 PGPR were selected as 5 phosphate solubilizers (P 45, P 49, P 50, P 51 and P 53), 3*Azospirillium* (P 03, P 20, P 28), 1 *Azotobacter* (P 27)and 1 *Pseudomonas* (P 44). Morphologically the colonies were studied on the basis of colony color, shape, elevation, and margin and staining character.

Biochemical characterization

Biochemical characterization of the selected cultures was assessed by using Hi- assorted biochemical test kits and characterized on the basis of carbohydrates utilization and enzyme activities so as to check the identical variation among the isolates. The individual culture was inoculated in each 25 ml selective broth and incubated for 3-4 days. After attaining maximum log phase, each individual culture was inoculated in the wells at 0.5 ml and

incubated for 18-24 hrs. After incubation, carbohydrate utilization was recorded on the basis of change in color and interpreted from the interpretive chart provided along with. However, the enzyme activities *viz.* Deamination and Nitrate reduction was assessed by incorporating reagent provided with the Hi- assorted biochemical test kits and the color change was interpreted from the chart provided along with.

Urease activity

Urease is the enzyme which hydrolyzes urea into carbon dioxide and ammonia. 10 selected bacterial cultures were tested in the urea test broth containing phenol red pH indicator (Brink, 2010). An increase in pH due to the production of ammonia resulted in color change from yellow (pH 6.8) to bright pink (pH 8.2) indicated positive urease activity and culture with yellow color indicated negative urease activity

HCN production

The production of HCN was tested in TSA slant amended with Glycine @ 4 g/lt (Bakker *et al.,* 1987). The slants were properly sealed, incubated for 4 days at 30±1°C to maintain gaseous metabolites produced by the test isolates and allow chemical reaction. The appearance of yellow to dark brown color of the filter paper strip indicated positive HCN production.

IAA Production

IAA is the most naturally occurring auxin which is the product of L-tryptophan metabolism produced by the PGPR. The selected PGPR cultures were tested for IAA production by spotting onto the Luria Bertani agar medium (LB) amended with L-tryptophan. The inoculated petriplates was overlaid with 82 mm diameter disk of nitrocellulose membrane and incubated at 30±1°C for 2-4 days at inverted position until the colony reached 2 mm on the surface of membrane filter. The membrane was removed from the plate and treated with salkowski reagent (2% of 0.5 M $Fecl_3$ in 35% Percloric acids). Formation of red halo within the membrane immediately surrounding the colony indicated positive for IAA production (Bric *et al.,* 1991).

Siderophore

Siderophores was detected by chrome azurolsulfonate (CAS) assay (Schwyn and Neilands, 1987). This assay uses an iron-dye complex which changes color on loss of iron. Siderophores, which have more affinity for the iron, removed the iron resulting in a change in the dye from blue to yellow/orange/purple.

Biocontrol activity against *Fusarium*sp

Antimicrobial activity of the 10 selected isolates against the fungal pathogen *Fusarium* sp. were screened out by dual culture technique (Rabindran and Vidyasekaran, 1996) in potato dextrose agar (PDA) media. The zone of mycelial growth of test pathogen was observed after5-7 days of incubation at 28±2°C and calculated in percent inhibition of the pathogen using the formula:

$$I = \frac{100(C - T)}{C}$$

Where, I = percentage inhibition of mycelia growth, C= growth of the pathogen in the control plate (cm) and T= growth of the pathogen in dual culture (cm)

Biocontrol activity against *Ralstonia solanacearum*

The ability to inhibit the growth of *Ralstonia solanacearum* by the bacterial isolates was

also checked out by dual culture assay (Sharma and Kaur, 2010). *R. solanacearum* was allowed to grow in sterilized Triphenyl Tetrazolium Chloride broth. Sterilized cotton swab was dipped into it while pressing against the inside wall of the flask above the fluid to remove excess inoculum and streaked over sterilized TTC agar medium. The bacterial isolates were spotted on the plate already having a lawn of pathogenic bacteria and incubated for 48 hrs at 30±1°C. The zone of inhibition around the isolates was measured and recorded in mm diameter.

Ammonia production

Ammonia production by the bacterial isolates was determined by Nessler method (Jackson, 1973). Each isolates was grown in 50 ml peptone water and incubated at 30°C. Centrifuged at 10,000 rpm for 10 min after incubation and to 1 ml supernatant was added to 2 ml of 10% sodium tartarate along with 1 ml of 1% gum acacia. To this solution 5 ml of Nessler's reagent was added and made up the volume to 50 ml and allowed to stand until yellow/brown color developed. The intensity of yellow/brown color was measured using UV-VIS spectrophotometer at 410 nm. The quantity of ammonia produced was calculated from the standard curve prepared with known concentration of ammonium chloride and expressed as mg l^{-1}.

Nitrogenase enzyme (Acetylene reduction Assay)

Nitrogenase activity was estimated by sensitive and inexpensive acetylene reduction assay (ARA) which was measured colorimetrically by the method described by Larue and Kerz (1973). The nitrogenase enzyme has also capacity to reduce Acetylene to Ethylene which can be measured indirectly either by using GC or colorimetrically. Overnight LB medium grown culture (1.5 ml) was spun and pellets washed carefully with sterilized distilled water to remove source of combined N, for further removal of traces the pellet was suspended in 100 µl of phosphate buffer saline. 10µl from this inoculum was added in 3 ml semi solid NFB medium in a 10 ml culture tube and the tubes were sealed with subba seal and incubated for 3 days. After incubation, 1.5 ml oxidant solution (80 ml of 0.05 M $NaIO_4$ + 10 ml of 0.005M $KMnO_4$, at pH 7.5) was added in the culture tube and sealed. 10% air from the culture tube was withdrawn and replaced with 10% acetylene gas. The suspension was agitated vigorously on a rotary shaker at 300 rpm for 90 min at room temperature. Thereafter, the subba seal was removed and 250µl of 4 M $NaAsO_2$ plus 250µl of 4 M NH_2SO_4 solution was added thoroughly to destroy excess oxidant. To this, 1 ml of Nash reagent (150g Ammonium acetate, 2ml of acetyl acetone diluted to one litre) was added and intensity of red color was measured after 60 min using UV-VIS spectrophometer at 412 nm wavelength. The quantities of nitrogenase enzyme produced was extraplotted from the standard curve prepared from the known concentration of C_2H_4 and expressed the quantity of C_2H_4 in µmol C_2H_4 $ml^{-1}hr^{-1}$.

The data obtained were analysed through completely randomized design and two factorial completely randomized design. The significance of variance was determined by calculating the respective F-value and significance of specific means differences was determined by calculating the critical differences (C.D) at 5% level of significance.

Results and Discussion

Diazotrophic PGPR not only fixed atmospheric nitrogen but also produced desired multiple PGP traits which could be used as efficient biofertizer and biocontrol agent (Sharma *et al.*, 2013; Shrivastava *et al.*,

2013 and 2014; Gururani *et al.,* 2014). 10 Diazotrophic PGPRidentified as P-Solubilizers, *Psuedomonas*, *Azotobacter*, *Azospirillium* cultures were screened out for ammonia production, Proteolytic activities, IAA production, Urease activities, HCN production, Antimicrobial activities, Siderophore production and Nitrogenase enzyme (ARA). Morphologically characterization shows the Diazotrophic PGPRcultures were non identical to each other (Table 1). Table 1 showed that all cultures were negative on Citrate utilization, Phenylalanine deamination and H_2S production but positive on utilization of Lysine and Ornithine. Some of the cultures could utilize Glucose, Arabinose and Sorbitol as carbon sources for instances cultures P 03, P 20, P 49 and P 51 but none of the cultures used adonitol and lactose as carbon source. Therefore, the results indicated that the cultures were non identical to each other which suggest species or strain variation among the test isolates in terms of utilization of different carbohydrates.

Allthe diazotrophic PGPR showed positive urease activity with varied intensity. Thus, the diazotrophic PGPR are efficiently capable to hydrolyze urea which also raised the pH and helps the diazotrophs to fix atmospheric nitrogen, enhances the rate of mineralization and nitrification (Nelson and Mele, 2006; Goswami *et al.,* 2015). Hydrogen cyanide (HCN) is a volatile, secondary metabolite formed from glycine associated with the plasma membrane of certain rhizobacteria by the action of HCN synthetase enzyme and inhibited soil borne phytopathogen by disrupting the electron transport in the living cell which caused death of pathogen (Blumer and Haas, 2000). The recent investigation, all Diazotrophic PGPR isolates were HCN producer with varied intensity of the production (Table 2). IAA is one of the most natural physiologically active auxins and PGPR are well-known for their ability to produce auxins which result in pronounce effects on plant growth. In the present investigation (Table 2), IAA production was recorded highest in culture P 03, P 49, P 20 and P 50 as compared to P 27, P 28, P 44, P 45, P 51 and P 53 based on intensity of color. However, the results showed that all the test cultures were able to produce IAA with the varied intensity of red halo among the isolates. Siderophores are low molecular weight compounds with high affinity for ferric iron produced by microbes and suppressed the growth of the pathogen by scavenge iron from the mineral phases (Sharma and Johri, 2003; Gururani *et al.,* 2014). 5 out of 10 diazotophic PGPR were siderophore producer where cultures P 28 and P 44 recorded the highest halo zone with 20 mmin CAS medium (Table 2). 8 of the selected diazotrophic bacteria were capable to suppress *Fusarium*sp (Table 2) with fungal mycelial inhibit percent ranges from 42.96 to 55.55% over control.

Culture P 49 (55.55%) record the maximum mycelial inhibition and culture P 28 (42.96%) recorded the lowest mycelial growth inhibition (Tabli *et al.,* 2014). However, no mycelial inhibition was recorded with cultures P 45 and P 53 (Table 2). 6 PGP cultures (P 03, P 20, P 44, P 45, P 49 and P 53) out of 10 were able to suppress against *Ralstonia solanacearum* with inhibition zone range from 15 mm to 21 mm in diameter. Among test cultures, P 45 and P 44 recorded maximum and minimum clear zone of inhibition respectively. Thus, the inhibition on test pathogen may be due to antimicrobial activities such as HCN and siderophorewhich suppress the growth of pathogen (Table 2) (Abidin *et al.,* 2016; Hassan *et al.,* 2017). Nitrogenase is a metallo enzyme biologically bound which catalyzes the reductive breakage of the very strong triple bond of N_2 to generate NH_3 an accessible form of nitrogen to the crop plant.

Table.1 Morphological and Biochemical characterization of diazotrophic PGPR cultures

Well no.	Test	P 03	P 20	P 27	P 28	P 44	P 45	P 49	P 50	P 51	P 53
A	**Morphological characterization of diazotrophic PGPR cultures**										
1	Color	White	White	Creamish white	Yellowish white	Greenish yellow	Creamish white	White	Creamish white	Creamish white	White
2	Margin	Entire	Entire	Entire	Entire	Entire	Serrated	Serrated	Entire	Entire	Entire
3	Shape	circular	Irregular	Circular	Irregular	Circular	Circular	Circular	Irregular	Circular	Circular
4	Elevation	Convex	Convex	Convex	Flat	Convex	Raised	Pulvinate	Convex	Convex	Umbonate
5	Gram reaction	-	-	-	-	-	-	+	-	-	-
B	**Biochemical characterization of diazotrophic PGPR cultures**										
1	Citrate utilization	-	-	-	-	-	-	-	-	-	-
2	Lysine utilization	+	+	+	+	+	+	+	+	+	+
3	Ornithine utilization	+	+	+	+	+	+	+	+	+	+
4	Urease	+	+	+	+	+	+	+	+	+	+
5	Phenylalanine deamination	-	-	-	-	-	-	-	-	-	-
6	Nitrate reduction	+	-	+	-	+	+	+	-	+	-
7	H_2S production	-	-	-	-	-	-	-	-	-	-
8	Glucose	+	+	-	-	+	-	+	-	-	+
9	Adonitol	-	-	-	-	-	-	-	-	-	-
10	Lactose	-	-	-	-	-	-	-	-	-	-
11	Arabinose	+	+	-	+	-	-	+	+	+	-
12	Sorbitol	+	-	+	-	-	-	+	-	+	-

Table.2 PGP activity produced by diazotrophic PGPR cultures

Sl. No.	Isolates	Siderophore activity Diameter of red/yellow /purple halo (mm)	Mycelial growth inhibition Against *Fusarium* sp (%)	Zone of inhibition against *Ralstonia* sp (mm diameter)	Urease activity	HCN Production	IAA Production	Ammonia (mgl^{-1})	C_2H_4 ($\mu mol\ ml^{-1}hr^{-1}$)
1	P 03 (*Azospirillium*)	-	43.71	18.66	+	+	+	309.05	11.00
2	P 20 (*Azospirillium*)	18	50.00	19.00	+	+	+	310.05	12.06
3	P 27 (*Azotobacter*)	-	45.55	-	+	+	+	302.82	9.04
4	P 28 (*Azospirillium*)	20	42.96	-	+	+	+	170.11	9.09
5	P 44 (*Pseudomonas*)	20	51.55	15.00	+	+	+	275.05	8.59
6	P 45 (PSB)	-	-	21.00	+	+	+	218.29	5.01
7	P 49 (PSB)	16	55.55	19.00	+	+	+	293.45	10.02
8	P 50 (PSB)	-	44.44	-	+	+	+	112.34	9.00
9	P 51 (PSB)	18	53.33	-	+	+	+	267.66	9.36
10	P 53 (PSB)	-	-	18.5	+	+	+	260.5	7.10
11	Control							81.05	2.35
							S.ED (±)	0.394	0.370
							$C.D_{0.05}$	0.817	0.768

Nitrogenase enzyme plays an important role in the fixing atmospheric nitrogen (Sudipta *et al.,* 2014). 10 diazotrophic bacterial isolates were significantly produced nitrogenase activity with a varying range of 5 -12.6 µmol C_2H_4 $ml^{-1}hr^{-1}$ over control (Table 2). The highest nitrogenase activity was recorded in culture P 20 (12.608 µmol $C_2H_4ml^{-1}hr^{-1}$) followed by culture P 03 (11 µmol C_2H_4 $ml^{-1}hr^{-1}$), P 49 (10.02 µmol C_2H_4 $ml^{-1}hr^{-1}$), P 51 (9.36 µmol C_2H_4 $ml^{-1}hr^{-1}$). On the contrary culture P 45 recorded lowest nitrogenase activity (5.012 µmol C_2H_4 $ml^{-1}hr^{-1}$). Banik *et al.,* 2016; Ritu *et al.,* 2017; Hassan *et al.,* 2017) reported 9.36 to 155.83 nmol C_2H_4 $mg^{-1}h^{-1}$ nitrogenase activity of 35 rhizobacteria by ARA technique and interpreted as greater the activity of nitrogenase enzyme, greater the ability of fixing atmospheric N_2. Similarly, all Diazotrophic PGPR isolates showed ammonia production ranging from 112.34 to 310.05 mg l^{-1} over control (Table 2). The production of ammoniawas recorded significantly the highest in culture P 20 (310.05 mg l^{-1}) followed by culture P 03 (309.05 mg l^{-1}).

The present investigation can be concluded that the selected diazotrophic isolates have their potential to be used as biofertilizers, phytostimulators and biocontrol inoculants. Diazotrophic isolates P 20 (*Azospirillium*), P 49 (PSB) and P51 (PSB) has been found to have edge over other bacterial cultures giving direct or indirect benefit to the plant growth. However, greater attention may be paid to studies and application of new combination of diazotrophic bacteria and other PGPR in field conditions with diverse crops to get a clear picture about the role of diazotroph.

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