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Occurrence, distribution and N_2 fixing ability of diazotrophic bacterial isolates from different rice production systems

S. KANIMOLI, M. KARTHIK AND K. KUMAR

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
Email : kanimicro76@gmail.com

Rice is now grown in different production systems *viz*., lowland, system of rice intensification (SRI) and aerobic rice. Since the agronomic conditions prevailing in these systems are different, the nitrogen fixing ability of the diazotrophic bacteria associated with these production systems may also vary. Hence, heterotrophic diazotrophs were isolated from the rhizosphere soils of rice grown in three different rice growing systems to assess their N_2 fixing potential. The diazotrophs were isolated from five different locations in Tamil Nadu.The different diazotrophs isolated belonged to the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia* and *Pseudomonas*. A total of hundred and ten isolates obtained were subjected to acetylene reduction assay (ARA) and ninety eight isolates recorded significant amount of nitrogenase activity in the range of 185.73 to 3794.55 nmoles of ethylene mg of protein^{-1} h^{-1}. Maximum nitrogenase activity was recorded by *Derxia* (3794.55 nmoles of ethylene mg of protein^{-1} h^{-1}) isolated from Trichy (lowland). Among the three different rice production systems, isolates obtained from lowland rice (*Derxia* – 3794.5 nmoles of ethylene mg of protein^{-1} h^{-1}) recorded higher nitrogenase activity followed by aerobic rice isolate (*Pseudomonas* - 2194.89 nmoles of ethylene mg of protein^{-1} h^{-1}) and SRI rice isolate (*Azotobacter* - 1971.85 nmoles of ethylene mg of protein^{-1} h^{-1}). The results revealed marked variation in the ARA of the diazotrophic isolates obtained from lowland, SRI and aerobic rice.

Key words : Acetylene reduction assay, Diazotrophs, Heterotrophs, Lowland, Aerobic, SRI

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INTRODUCTION

Rice after wheat is the most important crop and has a very valuable role in feeding people in the world (Khourgami *et al*., 2012) . In the next three decades, the world will need to produce about 60 per cent more rice than today's global production to feed the extra billion people. Nitrogen is the major nutrient limiting the high yield potential of modern rice cultivars. Development of fertilizer responsive varieties, coupled with the realization of the importance of nitrogen by farmers have led to high rates of N fertilizer use on rice. But unfortunately a substantial amount of the N fertilizer is lost through different mechanisms causing environmental pollution problems. Utilization of biological N fixation (BNF) technology can decrease the use of N fertilizer, reducing the environmental problems to a considerable extent. BNF technologies must be economically viable, ecologically sound and socially acceptable to be successful (Ladha and Reddy, 2003). In agricultural soils, except for anthropogenic sources, diazotrophic communities are the main source of nitrogen. Biological fixation offers a non-polluting source of nitrogen and could improve crop production and decrease the global use of synthetic fertilizers. Nonsymbiotic bacterial diazotrophs can promote economic and environmental benefits including increased income from high yields, reduced fertilizer costs and reduced emissions of the greenhouse gas N_2O, as well as reduced leaching of NO_3 to ground water (Kennedy *et al*., 2004).

Diazotrophic bacteria are known to directly and indirectly affect plant growth, directly through a substantial contribution of BNF to N acquisition of the plant and indirectly through the synthesis and export of organic substances like phytohormones that enhance root growth. The free-living and plant associated bacteria are ubiquitous in soil, but their diversity and contribution to N-input have not been fully understood (Bürgmann *et al*., 2004). Irrigated

or lowland rice in Asia is typically transplanted into puddled fields. Land preparation consists of soaking followed by ploughing and harrowing of saturated soil. After crop establishment, the fields are kept submerged with 5-10 cm of water. In SRI paddy cultivation, the fields are not flooded but the soil is kept moist during vegetative phase and later one inch water depth is maintained. SRI requires only about half the water as normally applied in irrigated rice. Aerobic rice is a new way of growing rice that needs less water than lowland rice. It is grown like an upland crop such as wheat, in soil that is not puddled, flooded, or saturated. The soil is, therefore, "aerobic" or with oxygen throughout the growing season, as compared to traditional flooded fields, which are "anaerobic." Hence, attempts were made to isolate, identify and evaluate the N_2 fixing potential of diazotrophic bacteria from the rhizosphere of rice from different location and different rice production systems *viz.*, Lowland, SRI and aerobic grown at different locations of Tamil Nadu.

RESEARCH METHODOLOGY

Collection of soil sample :

The rhizosphere soil samples were collected from five different locations in Tamil Nadu *viz.*, Coimbatore (Thondamuthur – Ikaraipoluvampatty, Saadivayal and Muttathuvayal), Pollachi (Ko ttur, Somandurai and Ponnapuram), Aduthurai (Melamaruthuvakudi, Kelamaru - thuvakudi and Avainyapuram), Trichy (Poovalur, Vaaladi and Maandurai) and Kililulam (Morappanadu, Naanalkadu and Kongaraiyarkuruchi). Rhizosphere soil samples were collected carefully by uprooting the root system and placed in a cool box for transport and stored at 4 ^{0}C.

Isolation and enumeration of diazotrophic bacteria :

Diazotrophic bacteria were enumerated and isolated by following standard agar plate count method (Allen, 1953). The different N_2 free media used were Waksman No 77 medium for *Azotobacter*, Becking's medium (Becking,1961) for *Beijerinckia*, Nitrogen- free glucose mineral medium for *Derxia* (Becking,1981) and King's B medium (King *et al*., 1954) for *Pseudomonas*. In case of *Azospirillum* most probable number (MPN) technique (Cochran, 1950) was followed by using nitrogen free malic acid semi solid medium (Dobereiner, 1980).

One gram of soil from each sample was aseptically weighed, transferred to 100 ml sterile water blank and shaken (120 rpm) for 30 min to get 10^{-2} dilution, after thorough shaking, one ml of diluent from 10^{-2} dilution was transferred to 9 ml water blank to get 10^{-3} dilution. Likewise, the sample was diluted serially with 9 ml water blanks until the appropriate dilution was obtained. Aliquots (1 ml) from the serially diluted samples (10^{-3} to 10^{-6}) were added to five different N-free media in Petri plates and kept in an incubator at 30°C. used for isolation and enumeration. Five days after incubation, colonies growing on N-free media were counted and the population of the diazotrophic bacteria estimated by using the following formula :

$$\text{Population of diazotrophic bacteria on dry weight basis} = \frac{\text{No. of colony forming unit x dilution factor}}{\text{Weight of the sample}} \times \frac{100}{100 - \text{Moisture \% of the sample}}$$

Colonies with similar colony characters were grouped according to their morphological characteristics. Single colonies were picked from the Petri dishes and sub-cultured several times to obtain pure cultures. Stock cultures were made in nutrient broth containing 50 per cent (w/v) glycerol and stored at –80°C.

Characterization of diazotrophic bacterial isolates :

Physiological and biochemical characters of the bacterial isolates were examined according to methods described in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). The isolates were characterized for the following traits: color, pigment, form, elevation, margin and texture. Motility and morphology were evaluated by performing phase contrast microscopy. The Gram staining was performed for checking the Gram reaction of the isolates as per standard procedures. The catalase activity was performed as per the method described by Gerhardt *et al.* (1981).

Acetylene reduction assay :

The nitrogen fixing capacity of the isolates was evaluated by acetylene reduction assay in the gas chromatograph (Chemito GC 7610) following the standard procedure (Burris, 1974). Twenty five ml of the respective broth was dispensed in 100 ml vials and sterilized. One ml of standard inoculum of the isolated cultures were added to the vials, mixed well and incubated for 72 hrs. Then cotton plugs were replaced with rubber stoppers and fixed with aluminum caps. Seventy five ml of air from the head space of the vial was withdrawn and nitrogen gas was flushed to remove the excess oxygen and 5ml of pure acetylene gas were injected into the vial and incubated at 37° C for 48 hrs. After incubation period, 0.2 ml of gas sample was withdrawn using a sterile disposable microsyringe and injected into the gas chromatograph (Chemito GC 7610) fitted with a poropak N column and FID detector. The column temperature was maintained at 120°C and injector and detector temperature were maintained at 150°C. The peak height was measured and nitrogenase activity was estimated and expressed as n moles of ethylene formed $h^{-1}mg^{-1}$ protein. Those cultures which showed high nitrogenase activity were selected for further studies.

Statistical analysis :

The data were subjected to analysis of variance

(ANOVA) and significance at the 5 per cent level was tested using SAS package, Version 8.2 (SAS, 2001).

RESEARCH FINDINGS AND ANALYSIS

The experimental findings obtained from the present study have been discussed in following heads:

Occurrence and distribution of diazotrophs from rice rhizosphere soil :

Several factors *viz.*, root morphology, the stage of plant growth, root exudates, and the physical and chemical properties of the soil are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere. Previous isolations of nitrogen fixing bacteria have revealed a broad diversity of diazotrophs to inhabit the crop rhizosphere (Vessey, 2003) and this study surveyed the rhizosphere soil of different rice growing system in different parts of Tamil Nadu for the presence of nitrogen fixing bacteria. Among the five major diazotrophs the population of *Pseudomonas* was found to be predominant at all locations and maximum population was recorded as (8.00×10^{10}) cfu/ g at Killikulam in SRI system on 15 DAT. It was followed by *Azospirillum*, *Azotobcter,Beijerinckia* and *Derxia* (Table 1).

In this study, total of 150 diazotrophic isolates which included the genera of *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia* and *Pseudomonas* were isolated from different locations of Tamil Nadu. Based on morphological

Table 1: Enumeration of major diazotrophs in the rhizosphere of rice at different rice growing areas in Tamil Nadu

Coimbatore (Thondamuthur)	15th DAT			30th DAT			45th DAT		
	LC_1I	SC_1S_1	AC_1M_1	LC_1I	SC_1S_1	AC_1M_1	LC_1I	SC_1S_1	AC_1M_1
Azospirillum**	$0.43x10^4$	$0.95 x10^4$	$0.84 x10^4$	$2.10 x10^4$	$2.50 x10^4$	$1.10 x10^4$	$1.30 x10^4$	$1.7 x10^4$	$1.50 x10^4$
Azotobacter*	$10.00 x10^3$	$7.00 x10^3$	$5.00 x10^3$	$15.00 x10^3$	$18.00 x10^3$	$12.00 x10^3$	$20.00 x10^3$	$22.00 x10^3$	$19.00 x10^3$
Beijerinckia*	$5.00 x10^3$	$4.00 x10^3$	$2.00 x10^3$	$8.00 x10^3$	$7.00 x10^3$	$5.00 x10^3$	$10.00 x10^3$	$12.00 x10^3$	$14.00 x10^3$
Derxia*	$3.10 x10^3$	$2.40 x10^3$	$5.10 x10^3$	$4.80 x10^3$	$5.30 x10^3$	$3.60 x10^3$	$5.20 x10^3$	$4.70 x10^3$	$2.80 x10^3$
Pseudomonas*	$7.30 x10^6$	$8.20 x10^6$	$6.40 x10^6$	$11.10 x10^6$	$10.40 x10^6$	$9.00 x10^6$	$17.00 x10^6$	$12.30 x10^6$	$10.30 x10^6$
Coimbatore (Pollachi)	LC_2K_1	SC_2S_2	AC_2P_1	LC_2K_1	SC_2S_2	AC_2P_1	LC_2K_1	SC_2S_2	AC_2P_1
Azospirillum**	$0.13 x10^6$	$0.16 x10^6$	$0.80 x10^6$	$0.95 x10^6$	$1.20 x10^6$	$1.50 x10^6$	$1.80 x10^6$	$2.10 x10^6$	$0.90 x10^6$
Azotobacter*	$8.10 x10^4$	$7.30x10^4$	$10.10 x10^4$	$12.30 x10^4$	$16.10 x10^4$	$20.30 x10^4$	$22.10 x10^4$	$26.30 x10^4$	$28.10 x10^4$
Beijerinckia*	$4.20 x10^3$	$3.60 x10^3$	$5.10 x10^3$	$5.80 x10^3$	$6.10 x10^3$	$4.30 x10^3$	$5.30 x10^3$	$4.80 x10^3$	$3.60 x10^3$
Derxia*	$3.60 x10^3$	$2.40 x10^3$	$1.50 x10^3$	$2.40 x10^3$	$3.60 x10^3$	$2.80 x10^3$	$3.00 x10^3$	$5.40 x10^3$	$4.10 x10^3$
Pseudomonas*	$10.10 x10^6$	$8.50 x10^6$	$11.20 x10^6$	$16.60 x10^6$	$20.10 x10^6$	$18.60 x10^2$	$11.60 x10^2$	$12.40 x10^2$	$20.60 x10^2$
Aduthurai	LAM_2	SAK_2	AA_1A_2	LAM_2	SAK_2	AA_1A_2	LAM_2	SAK_2	AA_1A_2
Azospirillum**	$0.17 x10^5$	$0.20 x10^5$	$0.01 x10^5$	$0.20 x10^5$	$0.20 x10^5$	$0.31x10^5$	$0.82 x10^5$	$3.10 x10^5$	$0.60 x10^5$
Azotobacter*	$1.66 x10^3$	$3.00 x10^3$	$5.00 x10^3$	$3.00x10^3$	$12.0 x10^3$	$14.00 x10^3$	$7.00 x10^3$	$16.00 x10^3$	$17.00 x10^3$
Beijerinckia*	$0.33 x10^3$	$0.15 x10^3$	$0.56 x10^3$	$0.60x10^3$	$1.00 x10^3$	$0.77x10^3$	$0.66 x10^3$	$1.20 x10^3$	$1.00 x10^3$
Derxia*	$0.96 x10^3$	$0.33 x10^3$	$0.37 x10^3$	$2.43x10^3$	$1.00 x10^3$	$1.20 x10^3$	$1.40 x10^3$	$1.60 x10^3$	$2.00 x10^3$
Pseudomonas*	$2.00 x10^6$	$3.50 x10^6$	$3.00 x10^6$	$3.00x10^4$	$8.00 x10^4$	$4.50 x10^4$	$7.0 x10^6$	$11.5 x10^6$	$10 x10^6$
Trichy	LTP_2	STV	ATM_3	LTP_2	STV	ATM_3	LTP_2	STV	ATM_3
Azospirillum**	$0.90 x10^5$	$3.30 x10^5$	$2.30 x10^5$	$1.10x10^5$	$1.40x10^5$	$7.80x10^5$	$1.40 x10^5$	$1.60 x10^5$	$2.00 x10^4$
Azotobacter*	$5.00 x10^4$	$3.50 x10^4$	$1.50 x10^4$	$1.50x10^4$	$2.50x10^4$	$2.50x 10^4$	$2.00 x10^4$	$3.30 x10^6$	$7.00 x10^6$
Beijerinckia*	$7.00 x10^4$	$4.70 x10^5$	$2.30 x10^5$	$7.00 x10^4$	$1.00x10^5$	$2.00x10^5$	$6.30 x10^4$	$9.6 0x10^4$	$1.30 x10^5$
Derxia*	$1.00 x10^3$	$3.50 x10^4$	$3.50 x10^3$	$1.00x10^3$	$3.00 x10^4$	$2.50x10^3$	$5.00 x10^4$	$3.20 x10^4$	$7.00 x10^3$
Pseudomonas*	$1.10 x10^6$	$2.10 x10^6$	$1.40 x10^6$	$4.00 x 10^6$	$9.00x10^6$	$5.00x10^6$	$1.30 x10^6$	$3.00 x10^6$	$2.30 x10^6$
Killikulam	LKM_4	SKN	AKK_3	LKM_4	SKN	AKK_3	LKM_4	SKN	AKK_3
Azospirillum**	$0.10x10^5$	$0.4 0x10^5$	$0.70 x10^5$	$2.20 x10^5$	$2.10 x10^5$	$0.60 x10^5$	$2.00 x10^5$	$2.40x10^5$	$4.20 x10^5$
Azotobacter*	$11.00 x10^3$	$12.00 x10^3$	$9.00 x10^3$	$16.00 x10^3$	$24 .00x10^3$	$10.00 x10^3$	$38.00 x10^3$	$29.00 x10^3$	$19.00 x10^3$
Beijerinckia*	$4.00 x10^3$	$4.00 x10^3$	$2.00 x10^3$	$9.00 x10^3$	$10.00 x10^3$	$2.00 x10^3$	$12.00 x10^3$	$16.00 x10^3$	$0.50 x10^3$
Derxia*	$16.00 x10^3$	$21.00 x10^3$	$11.00 x10^3$	$22.00 x10^3$	$29.00 x10^3$	$15.00 x10^3$	$34.00 x10^3$	$32.00 x10^3$	$28.00 x10^3$
Pseudomonas*	$16.00 x10^6$	$8.00 x10^{10}$	$12.00 x10^6$	$29.00 x10^6$	$14.00 x10^6$	$10.00 x10^6$	$41.00 x10^6$	$19.00 x10^6$	$15.00 x10^6$

* Population in cfu g^{-1} soil on oven dry basis. **Poulation in no.of cells on oven dry basis

L -Lowland, S – SRI, A – Aerobic, C_1- Coimbatore (Thondamuthur), C_2 – Coimbatore Pollachi), A_1- Aduthurai, A_2 – Avainyapuram, I – Ikaraipoluvampatty, K – Killikulam, K_1- Kottur, K_2 – Kelamaruthuvakudi, K_3 – Kongaraiyarkuruchi, S_1 – Saadivayal,S_2 – Somandurai, M_1- Muttathuvayal, M_2 – Melamaruthuvakudi, M_3- Maandurai, M_4- Morappanadu, N - Naanalkadu, P_1- Ponnapuram, P_2 – Poova

and biochemical characteristics hundred and ten isolates were selected and subjected to acetylene reduction assay. Though the ability to reduce acetylene is an indirect measure of N_2-fixation, it is specific for monitoring functional nitrogenase activity, and is indicative of N_2-fixing potential (Andrade *et al*., 1997). Hence, for further identification, screening and selection of prospective strains, ARA was used as a test for diazotrophy. Out of hundred and ten isolates, ninety eight isolates exhibited nitogenase activity. The maximum nitrogenase enzyme activity was recorded in *Derxia* (3794.55 nmoles of C_2H_4-^{1}mg of protein^{-1}hr) isolated from lowland, Trichy followed by *Azotobacter* (2463.12 nmoles of $C_2H_4 mg^{-1}$ of protein hr^{-1}) from Trichy (Lowland) (Table 2). This was confirmed by Keyeo *et al*. (2011) that diazotrophs have the ability to fix atmospheric nitrogen. Flooding a soil cuts off its oxygen supply. During the succession of anaerobic oxidation processes, the redox potential (Eh) of flooded soils will decrease as a result of the reduced products formed. Oxygen is depleted soon after flooding. Within a few hours, soil organisms use up the trapped oxygen and render the soil anaerobic. To grow and ward off toxins which are present in anaerobic soils, rice has evolved a genetically fixed system of transporting oxygen from shoot to roots. The system is only lightly less efficient in upland than in lowland rice (Huang *et al*., 2009). This remarkable characteristic of rice operates to its disadvantage when it is grown in aerobic soils.

Table 2: Acetylene reduction assay of different diazotrophic isolates from different rice growing areas in Tamil Nadu

Sr. No.	Name of the isolate	ARA – nmoles of ethylene mg^{-1} protein h^{-1}				
		Lowland	Name of the isolate	SRI	Name of the isolate	Aerobic
1.	$AsLC_1I$	1476.3	$AsSC_1I$	907.5	$AsAC_1M_1$	361.6
2.	$AsLC_2K_1$	1461.0	$AsSC_2S_2$	863.0	$AsAC_2P_1$	717.8
3.	$AsLAM_3$	577.7	$AsSAK_2$	1621.3	$AsAA_1I$	552.3
4.	$AsLTP_2$	1609.3	AsSTV	591.1	$AsATM_2$	1187.3
5.	As LKM_4	821.3	AsSKN	1056.9	$AsAKK_3$	1416.4
6.	$AbLC_1I$	1044.7	$AbSC_1I$	1480.6	$AbAC_1M_1$	1018.0
7.	$AbLC_2K_1$	764.4	$AbSC_2S_2$	572.6	$AbAC_2P_1$	394.4
8.	$AbLAM_3$	1422.2	$AbSAK_2$	1327.9	AbAAA	1740.4
9.	$AbLTP_2$	2463.1	Ab STV	1508.0	$AbATM_2$	865.4
10.	$AbLKM_4$	894.3	AbSKN	1971.8	$AbAKK_3$	376.3
11.	BeC_1I	1716.3	$BeSC_1I$	1702.4	$BeAC_1M_1$	1810.6
12.	$BeLC_2K_1$	956.4	$BeSC_2S_2$	1806.2	$BeAC_2P_1$	1613.1
13.	$BeLAM_3$	364.3	$BeSAK_2$	672.8	BeAAA	1180.8
14.	$BeLTP_2$	750.1	BeSTV	211.1	$BeATM_2$	539.6
15.	$BeLKM_4$	315.9	BeSKN	695.0	$BeAKK_3$	514.3
16.	$DeLC_1I$	512.1	$DeSC_1I$	1358.8	$DeAC_1M_1$	1013.2
17.	$DeLC_2$ K_1	863.2	$DeSC_2S_2$	756.4	$DeAC_2P_1$	1821
18.	$DeLAM_3$	965.4	$DeSAK_2$	1078.1	DeAAA	2055.5
19.	$DeLTP_2$	3794.5	DeSTV	891.7	$DeATM_2$	1915.1
20.	$DeLKM_4$	621.3	DeSKN	1021.3	$DeAKK_3$	946.7
21.	$PsLC_1I$	631.1	$PsSC_1I$	1452.1	$PsAC_1M_1$	1054.7
22.	$PsLC_2K_1$	946.3	Ps SC_2S_2	837.1	$PsAC_2P_1$	2194.8
23.	$PsLAM_3$	1112.5	Ps SAK_2	1368.7	Ps AA_1A_2	1249.3
24.	$PsLTP_2$	1023.1	Ps STV	1038.9	Ps ATM_2	941.3
25.	$PsLKM_4$	774.4	Ps SKN	700.9	Ps AKK_3	689.4
	S.E. $\pm$	159.3		134.5		149.4
	C.D. (P =0.05)	320.1		270.2		300.2

Az – Azospirillum, Ab – Azotobacter, Be – Beijerinckia, De – Derxia, Ps – Pseudomonas, L – Lowland, S – SRI, A – Aerobic,
C_1- Coimbatore (Thondamuthur), C_2 - Coimbatore (Pollachi), A_1- Aduthurai, A_2 - Avainyapuram, I – Ikaraipoluvampatty, K – Killikulam,
K_1- Kottur, K_2 – Kelamaruthuvakudi, K_3 – Kongaraiyarkuruchi, S_1 – Saadivayal,S_2 – Somandurai, M_1- Muttathuvayal, M_2 – Melamaruthuvakudi,
M_3- Maandurai, M_4- Morappanadu, N - Naanalkadu, P_1- Ponnapuram, P_2 – Poovalur.

Conclusion :

Several new free living diazotrophs have been reported in rice in the recent past and the exploitation of their potential will be the future strategy for sustained rice production. It has become the need of the hour to extend the molecular tools in conjunction with microbiological methods for analysing the genetic diversity of free living diazotrophs. More intensive research is essential to study the molecular diversity of diazotrophs from rhizospheric and non rhizospheric soil of rice.

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