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Studies on nitrogenase activity of diazotrophic isolates from different rice production systems

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Abstract: The present study was carried out to evaluate the nitrogen fixing ability of diazotrophs isolated from the rhizosphere soils of rice which were grown in three different rice growing systems. 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 a range of 185.73 to 3794.55 nmoles of ethylene mg of protein^{-1} h^{-1}. The highest 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 (*Pseudomonas* - 2194.89 nmoles of ethylene mg of protein^{-1} h^{-1}) and SRI (*Azotobacter* - 1971.85 nmoles of ethylene mg of protein^{-1} h^{-1}) rice isolates. The results revealed marked variation in the ARA of the diazotrophic isolates obtained from lowland, SRI and Aerobic rice. The nitrogenase activity of diazotrophs from rice fields have been reported earlier but the nitrogenase activity of diazotrophs from three different rice production systems from various parts of Tamil Nadu is reported for the first time from India.

Keywords: Acetylene reduction assay, Aerobic, Diazotrophs , Heterotrophs, Lowland

INTRODUCTION

Rice is the most important cereal crop. The primary source of rice is from Asia and India and also rainfed rice cultivation have been popular for about five thousand years. Rice after wheat is the most important crop and has a very valuable role in feeding people in the world**.** In the next three decades, the world will need to produce about 60% more rice than today's global production to feed the extra billion people. Biological nitrogen fixations (BNF) by diazotrophs play a significant role in nitrogen cycling and have drawn the attention of researchers because they represent one of the largest sources of nitrogen input to soil. Diazotrophs are present in the aerobic soil layers, rhizosphere, roots and stem bases of rice agro-ecosystems. They have the capacity to fix molecular nitrogen under microaerophilic conditions as a growth promoting substrate in nitrogen deficient environment. All the known diazotrophs possess *nif*H gene, which encodes the iron protein subunit of nitrogenase enzyme, and has been frequently used as a molecular marker to detect diazotrophs in environmental samples (Sarkar *et al*., 2014).

Nitrogen is the major nutrient limiting the high yield potential of modern rice cultivars. Development of fertilizer responsive varieties, coupled with the realization by farmers of the importance of nitrogen, has 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 (Rangjaroen *et al*., 2015). 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 there are little understanding of their diversity and contribution to N-input. The objective of this present study was to isolate and identify diazotrophic bacteria from the rhizosphere of rice from different loca-

tion and different rice production systems *viz.*, Low-land, SRI and Aerobic.

MATERIALS AND METHODS

Collection of soil sample: The rhizosphere soil samples were collected from five different locations in Tamil Nadu *viz*., Coimbatore (Thondamuthur – Ikaraipo-luvampatty, Saadivayal and Muttathuvayal), Pollachi (Kottur, Somandurai and Ponnapuram), Aduthurai (Melamaruthuvakudi, Kelamaruthuvakudi and Avainyapuram), Trichy (Poovalur, Vaaladi and Maan-durai) and Killilulam (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 of diazotrophic bacteria from rhizosphere soil: Diazotrophic bacteria were enumerated and isolated by following standard plate count method (Allen, 1953). The different N 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 for isolation.

Purification and labelling of isolates: 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% (w/v) glycerol and stored at –80°C.

Table 1. Acetylene reduction assay of *Azospirillum* from different rice growing areas in Tamil Nadu.

S.N.	Rice production system	Location	Village	Strain Name	ARA activity (nmoles of C_2H_4 mg^{-1} protein h^{-1})
1.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*As*AC_2P_1-1	717.8
2.	SRI	Killikulam	Naanalkadu	*As*SKN	1056.9
3.	*Azospirillum* (Az -204)				658.6
4.	Lowland	Aduthurai	Melamaruthuvakudi	*As*LAM_2 -1	1461.0
5.	Lowland	Coimbatore (Pollachi)	Kottur	*As*LC_2K_1- 1	756.2
6.	Aerobic	Trichy	Maandurai	*As*ATM_3-1	1187.3
7.	Lowland	Coimbatore (Pollachi)	Kottur	AsLC_2K_1 -2	1476.3
8.	SRI	Trichy	Vaaladi	*As*STV -1	900.4
9.	Lowland	Trichy	Poovalur	*As*LTP -1	482.9
10.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*As*LC_1I-1	1584.3
11.	Lowland	Trichy	Poovalur	*As*LTP -2	1609.3
12.	SRI	Coimbatore (Thondamuthur	Saadivayal	*As*SC_1S_1	907.5
13.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*As*AC_2P_1-1	361.6
14.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*As*LC_1I-1	577.7
15.	Aerobic	Trichy	Maandurai	*As*ATM_3 -2	877.6
16.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*As*AC_2P_1-2	730.3
17.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*As*AC_2P_1-3	519.7
18.	Aerobic	Coimbatore (Thondamuthur)	Muttathuvayal	*As*AC_2M_1-4	589.1
19.	Aerobic	Coimbatore (Thondamuthur)	Muttathuvayal	*As*AC_2M_1-5	863.0
20.	Aerobic	Killikulam	Kongaraiyarkuruchi	*As*AKK_3	1416.4
21.	Lowland	Killikulam	Morappanadu	*As*LKM_4	821.3
22.	Aerobic	Trichy	Maandurai	*As*ATM_3 -3	902.0
23.	Lowland	Aduthurai	Melamaruthuvakudi	*As*LAM_2 -2	588.5
24.	SRI	Trichy	Vaaladi	*As*STV -2	591.1
25.	Aerobic	Aduthurai	Avainyapuram	*As*AA_1A_2	552.3
	SEd				39.1
	CD (P =0.05)				78.6

Determination of nitrogenase activity of the isolates: The nitrogen fixing capacity of the isolates was evaluated for 115 diazotrophic isolates 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 which was 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 fitted with a poropak Q 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: Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 5% level was tested by Least Significant Difference (LSD) using SAS package, Version 8.2 (SAS, 2001).

RESULTS AND DISCUSSION

Isolation of diazotrophs from rice rhizosphere soil: The different diazotrophic population was isolated from rhizosphere soil of rice at different locations of Tamil Nadu. The current investigation demonstrated that the diazotrophs thrived well with maximum numbers in the rhizosphere soil of rice isolated from different locations of Tamil Nadu. Among the diazotrophs, population of *Pseudomonas* was found to be maximum followed by *Azospirillum* and *Azotobacter*. Kumar and Sugitha (2004) reported that the proportion of total diazotrophs to total heterotrophs was in the range of 12.39 to 20.65% in the rhizosphere of different rice cultivars and the distribution pattern of these diazotrophs was in the order of *Pseudomonas* > *Azospirillum* > *Azotobacter* > *Beijerinckia* > *Derxia* > *Klebsiella* > *Enterobacter*. The results of the present study are in conformity with earlier findings of Kumar and Sugitha. This high proportion of the diazotrophs in

Table 2. Acetylene reduction assay of *Azotobacter* from different rice growing areas in Tamil Nadu.

S.N.	Rice production system	Location	Village	Strain Name	ARA activity (nmoles of C_2H_4 mg^{-1} protein h^{-1})
1.	Aerobic	Trichy	Maandurai	*Ab*ATM$_3$	737.3
2.	Aerobic	Aduthurai	Avainyapuram	*Ab*AA$_1$A$_2$-1	1740.4
3.	Lowland	Aduthurai	Melamaruthuvakudi	*Ab*LAM$_2$ -1	782.3
4.	Lowland	Aduthurai	Melamaruthuvakudi	*Ab*LAM$_2$ -2	361.5
5.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*Ab*LC$_1$I-1	1047.7
6.	SRI	Aduthurai	Kelamaruthuvakudi	*Ab*SA$_1$K$_2$-1	681.8
7.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Ab*AC$_2$P$_1$-1	1018.0
8.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Ab*AC$_2$P$_1$-2	394.4
9.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Ab*AC$_2$P$_1$-3	764.4
10.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Ab*AC$_2$P$_1$-4	512.6
11.	Aerobic	Aduthurai	Avainyapuram	*Ab*AA$_1$A$_2$-2	627.4
12.	Lowland	Coimbatore Thondamuthur)	Ikaraipoluvampatty	*Ab*LC$_1$I-2	863.7
13.	Lowland	Aduthurai	Melamaruthuvakudi	*Ab*LAM$_2$ -3	1422.2
14.	SRI	Aduthurai	Kelamaruthuvakudi	*Ab*$_1$K$_2$ -2	1327.9
15.	SRI	Coimbatore (Thondamuthur	Saadivayal	*Ab*SC$_1$S$_1$	1480.6
16.	Lowland	Trichy	Poovalur	*Ab*LTP -1	1543.6
17.	SRI	Trichy	Vaaladi	*Ab*STV	1508.0
18.	SRI	Killikulam	Naanalkadu	*Ab*SKN	1971.8
19.	Lowland	Trichy	Poovalur	*Ab*LTP -2	2463.1
20.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*Ab*LC$_1$I-3	1320.0
21.	Aerobic	Killikulam	Kongaraiyarkuruchi	*Ab*AKK$_3$	376.3
22.	*Azotobacter chroococcum*				647.6
	SEd				48.8
	CD (P =0.05)				98.4

Table 3. Acetylene reduction assay of *Beijerinckia* from different rice growing areas in Tamil Nadu.

S.N.	Rice production system	Location	Village	Strain Name	ARA activity (nmoles of C_2H_4 mg^{-1} protein h^{-1})
1.	Aerobic	Trichy	Maandurai	*Be*ATM_3-1	438.4
2.	SRI	Coimbatore (Thondamuthur)	Saadivayal	*Be*SC_1S_1	1702.4
3.	SRI	Killikulam	Naanalkadu	*Be*SKN	695.0
4.	Aerobic	Aduthurai	Avainyapuram	*Be*AA_1A_2	1180.8
5.	SRI	Coimbatore (Pollachi)	Somandurai	*Be*SC_2S_2	1806.2
6.	Lowland	Trichy	Poovalur	*Be*LTP -1	750.1
7.	Aerobic	Killikulam	Kongaraiyarkuruchi	*Be*AKK_3	185.7
8.	Aerobic	Coimbatore (Thondamuthur)	Muttathuvayal	*Be*AC_2M_1	1810.6
9.	SRI	Trichy	Vaaladi	*Be*STV	211.1
10.	Aerobic	Trichy	Maandurai	*Be*ATM_3-1	539.6
11.	Lowland	Trichy	Poovalur	*Be*LTP -2	625.5
12.	Lowland	Killikulam	Morappanadu	*Be*LKM_4	315.9
13.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Be*AC_2P_1	1613.1
14.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*Be*LC_1I	1716.3
15.	Lowland	Aduthurai	Melamaruthuvakudi	*Be*LAM_2	364.3
	SEd				45.5
	CD (P =0.05)				92.9

the rhizosphere may be due the beneficial influence of root exudates. The findings of Xie and Yokota (2004) have specified that the number of nitrogen fixers is strongly governed by soil organic matter content, and is not significantly affected by water.

Nitrogenase activity of diazotrophs isolated from rhizosphere soil of rice: The confirmation test for nitrogen fixing ability was done by gas chromatographic analysis by acetylene reduction assay and the results are furnished in (Tables 1- 5).

A total of hundred and fifteen isolates obtained were subjected to Acetylene Reduction Assay (ARA) and ninety eight isolates recorded significant amount of nitrogenase activity in the range of 185.7 to 3794.6 nmoles of ethylene mg of $protein^{-1}$ h^{-1}. Maximum nitrogenase activity was recorded by *Derxia* (3794.5 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* - 2194.9 nmoles of ethylene mg of $protein^{-1}$ h^{-1}) and SRI rice isolate (*Azotobacter* - 1971.9 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.

The discovery that the nitrogenase enzyme responsible for nitrogen fixation also reduced C_2H_2 to C_2H_4 (Dilworth, 1966), a useful assay for the quantification of the nitrogen fixation process. For quantitative determinations of nitrogen fixation, $^{15}N_2$ techniques should be used, however, the acetylene reduction assay is still used widely, because it provides a highly sensitive and inexpensive way to quantify relative nitrogenase enzyme activity in nitrogen fixing samples.

Diazotrophic bacteria varied in acetylene reduction, which is an indirect measure of N-fixing potential while also being a specific means for monitoring of functional nitrogenase activity (Andrade *et al*., 1997 and Park *et al*., 2005).

All the 115 diazotrophic isolates were subjected to acetylene reduction assay out of which 99 isolates were able to fix atmospheric dinitrogen. The high acetylene reduction was recorded in the isolate *De*LTP with 3794.5 n moles of ethylene mg^{-1} protein h^{-1}. This was confirmed by Keyeo *et al*. (2011) that diazotrophs have the ability to fix atmospheric nitrogen. Flooding the 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*., 2012). This remarkable characteristic of rice operates to its disadvantage when it is grown in aerobic soils. Some strains isolated in this study showed relatively low ARA. Firstly, this could have been because the oxygen level was not controlled when incubating, and diazotrophs have a varying ability to tolerate oxygen (Barber and Evans, 1976; Postgate, 1998). Secondly, nitrogenase activity de-

Table 4. Acetylene reduction assay of *Derxia* from different rice growing areas in Tamil Nadu.

S.N.	Rice production system	Location	Village	Strain Name	ARA activity (nmoles of C_2H_4 mg^{-1} protein h^{-1})
1.	Lowland	Trichy	Poovalur	*De*LTP -1	551.6
2.	Lowland	Trichy	Poovalur	*De*LTP -2	3794.5
3.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*De*AC_2P	1821.0
4.	Lowland	Aduthurai	Melamaruthuvakudi	*De*LAM_2	965.4
5.	Aerobic	Trichy	Maandurai	*De*ATM_3-1	1913.1
6.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*De*LC_1I	512.1
7.	Lowland	Killikulam	Morappanadu	*De*LKM_4	621.3
8.	Aerobic	Aduthurai	Avainyapuram	*De*AA_1A_2	2055.5
9.	Aerobic	Trichy	Maandurai	*De*ATM_3-2	966.9
10.	SRI	Aduthurai	Kelamaruthuvakudi	*De*SA_1K_2	1078.1
11.	SRI	Trichy	Vaaladi	*De*STV	891.7
12.	SRI	Killikulam	Naanalkadu	*De*SKN	1211.5
13.	SRI	Coimbatore (Thondamuthur)	Saadivayal	*De*SC_1S_1	1358.8
14.	Aerobic	Coimbatore (Thondamuthur)	Muttathuvayal	*De*AC_2M_1	467.8
15.	SRI	Coimbatore (Pollachi)	Somandurai	*De*SC_2S_2	543.1
	SEd				61.5
	CD (P =0.05)				125.7

Table 5. Acetylene reduction assay of *Pseudomonas* from different rice growing areas in Tamil Nadu.

S.N.	Rice production system	Location	Village	Strain Name	ARA activity (nmoles of C_2H_4 mg^{-1} protein h^{-1})
1.	SRI	Coimbatore (Thondamuthur)	Saadivayal	*Ps*SC_1S_1	1368.7
2.	Lowland	Aduthurai	Melamaruthuvakudi	*Ps*LAM_2 -1	2401.7
3.	Aerobic	Aduthurai	Avainyapuram	*Ps*AA_1A_2-1	1249.8
4.	SRI	Coimbatore (Pollachi)	Somandurai	*Ps*SC_2S_2	837.1
5.	Aerobic	Coimbatore Thondamuthur)	Muttathuvayal	*Ps*AC_2M_1	1054.7
6.	Aerobic	Trichy	Maandurai	*Ps*ATM_3 -1	466.0
7.	Lowland	Aduthurai	Melamaruthuvakudi	*Ps*LAM_2 -2	760.3
8.	SRI	Killikulam	Naanalkadu	*Ps*SKN	700.9
9.	Lowland	Aduthurai	Melamaruthuvakudi	*Ps*LAM_2 -3	1112.5
10.	Aerobic	Trichy	Maandurai	*Ps*ATM_3 -2	941.3
11.	SRI	Trichy	Vaaladi	*Ps*STV	1038.9
12.	Lowland	Trichy	Poovalur	*Ps*LTP -1	576.4
13.	Lowland	Killikulam	Morappanadu	*Ps*LKM_4	774.4
14.	Lowland	Trichy	Poovalur	*Ps*LTP - 2	1023.1
15.	SRI	Aduthurai	Kelamaruthuvakudi	*Ps*A_1K_2 -1	1452.1
16.	Lowland	Trichy	Poovalur	*Ps*LTP - 3	635.6
17.	SRI	Aduthurai	Kelamaruthuvakudi	*Ps*A_1K_2 -2	574.0
18.	*Pseudomonas fluorescence* (Standard)				1498.1
19.	Aerobic	Aduthurai	Avainyapuram	*Ps*AA_1A_2-2	829.8
20.	Aerobic	Coimbatore (Pollachi)	Ponnapuram	*Ps*AC_2P	2194.8
21.	Aerobic	Aduthurai	Avainyapuram	*Ps*AA_1A_2-3	428.5
22.	Lowland	Coimbatore (Thondamuthur)	Ikaraipoluvampatty	*Ps*LC_1I	631.1
	SEd				46.5
	CD (P =0.05)				93.8

creases when nitrogen fixing bacteria are purified and cultured. Thirdly, diazotrophs show different nitrogen fixing ability when the composition of the cultural media is changed (Dobereiner, 1989). This was supported by a study (Shrestha *et al.*, 2007), which revealed that among all the carbon sources, almost all the diazotrophs preferred glucose for high nitrogen fixing activity and some preferred sucrose and other carbon sources like mannitol.

Conclusion

All the 115 isolates obtained from the rhizosphere of rice grown in three different production systems in different rice growing areas of Tamil Nadu were subjected to morphological and biochemical characterization for preliminary identification of isolates. All the isolates were Gram negative. In case of *Azospirillum,* the cells were spirillum and in *Azotobacter* some cells are found to be pleomorphic. The *Pseudomonas* isolates produced fluorescein pigment. Out of 115 diazotrophic isolates, 99 isolates recorded significant amount of nitrogenase activity in a range of 185.7 to 3794.5 nmoles of ethylene mg of protein^{-1} h^{-1}. The maximum nitrogenase activity was exhibited by isolate *De*LTP (3794.5 nmoles of ethylene mg of protein^{-1} h^{-1}). The nitrogenase activity of diazotrophs from rice fields have been reported earlier but the nitrogenase activity of diazotrophs from three different rice production systems from various parts of Tamil Nadu was novelty of study reported first time from India.

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