Madras Agric. J. 88 (10-12) : 618-621 October-December 2001
https://doi.org/10.29321/MAJ.10.A00386

Estimation of nitrogen fixation by immobilized anaerobic and facultative anaerobes using ^{15}N technique

P. MARIMUTHU AND S. GUNASEKARAN
Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore - 641 003

Abstract: Nitrogen fixation by the facultative and anaerobic nitrogen fixing *Klebsiella* and *Clostridium* was studied. After characterization cultures were immobilized with sodium alginate and inoculated. A pot culture experiment with IR 50 paddy indicated positive contribution by immobilized cells. The nitrogen fixing ability of the inoculated cultures were estimated using ^{15}N technique. The immobilized cells of *Clostridium* sp. and *Klesiella* sp. not only fixed the atmospheric N but also enhanced the nitrogen uptake by rice to the tune of 4.22 kg N ha^{-1} m^{-1} and 2.835 kg ha^{-1} m^{-1} respectively. *(Key words : Facultative anaerobes, Nitrogen fixation, Immobilization techniques).*

Rice is cultivated both in upland and lowland conditions. Dry, semi dry and wet cultivation of rice is common in India. Expansion of irrigating ayacut has increased the cultivation of wet land rice. Since the soil is saturated under flooded condition, the anaerobes prevail for the soil biocatalysis. Some of the soil anaerobes fix atmospheric nitrogen are of special interest in the flooded rice soil (Skinner, 1986). Nitrogen fixing microorganisms enrich nitrogen in submerged rice soils by atmospheric N_2 fixation (Grist, 1965). In flooded soil where rice is growing, dissolved nitrogen occurs in the root rhizosphere (Yoshida and Ancajas, 1971) and are amenable for N_2 transformation. Four techniques were used in the assay of N_2 fixation (net or gross). In order of increasing sensitivity of assay, they are i) total assay of Kjeldahal nitrogen (Bremner, 1965) ii) N_2 : Argon ratio (Koyamo, 1963, Wada *et al.* 1978) iii) ^{15}N enrichment (MacRae and Castro, 1967) iv) and C_2H_2 reduction (Lee and Watanabe, 1977 and Matsuguchi, 1979). An experiment was conducted in pot culture to study the nitrogen fixing ability of the immobilized cells of *Klebsiella* sp. and *Clostridium* sp using ^{15}N enrichment technique in paddy.

Materials and Methods

Organisations and Culture conditions

Nitrogen fixing anaerobe *Clostridium* and facultative anaerobe *Klesiella* were isolated using Hungates (1957) roll tube technique using the Hills medium (Bergerson, 1980). The individual colonies developed in the roll tubes were transferred to the vials containing nitrogen free medium and incubated under N_2 atmosphere. After desired growth, the cultures were purified and further assayed for fixing ability. These cultures were grown separately in different carbon sources. Further the purified cultures were immobilized with sodium alginate for ^{15}N studies. The volatile fatty acids produced by the cultures were estimated as described by Holdman *et al.* (1977).

Preparation of sodium alginate slurry

Sodium alginate of 3.5g was added to 100 ml of 0.1 per cent calcium chloride with constant stirring of the flask. The slurry obtained was homogenised by autoclaving. *Clostridium* sp. and *Klebsiella* sp, isolated from the flooded rice ecosystem were purified and characterized. These cultures were inoculated in sucrose medium (sucrose 20g; $Na_2H\ PO_4$ 10.4g; KH_2PO_4 3.4 g Fe(III) citrate 36g; $MgSO_4$ 30g; $CaCl_2.2H_2O$ 26g; $MnSO_4$ 0.3g; $Na_2MoO_4.2H_2O$ 66g; distilled water 1000ml) separately under nitrogen atmosphere and incubated anaerobically. They were grown until an optical density was reached to 0.6 at 600 nm. The cells were harvested by centrifugation at 15000 rpm for 15 min, washed with sterile 0.1 per cent saline, again centrifuged and resuspended in 0.1 per cent saline. The whole operation was performed by flushing nitrogen gas. Then the culture was added to sodium alginate slurry under nitrogen atmosphere and mixed thoroughly. Beads were prepared by passing the slurry through a tube connected to a peristaltic pump and allowing the drop to fall into a 4 per cent calcium chloride solution. The immobilized beads were transferred to a vial and stored under nitrogen atmosphere and used for pot culture studies.

Pot culture experiment

To study the nitrogen fixation by the facultative and anaerobic nitrogen fixing *Klebsiella* sp., and *Clostridium* sp. a pot culture experiment was laid out with IR 50 paddy. Twenty beads (CFU x 10^7 ml^{-1}) were inoculated into each plastic pot (35 cm length and 30 cm width), containing 6 kg of soil and water level was maintained 3 cm above soil. Randomized block design with four replications was employed for the study.

The plant samples were collected at 30 days after transplanting.

The following are the treatments

T_1 - N control

T_2 - 100 kg ^{15}N alone

T_3 - 100 kg ^{15}N + immobilized cells of *Clostridium*

T_4 - 100 kg ^{15}N + immobilized cells of *Klebsiella*

The ^{15}N urea 10 per cent atom excess supplied by Rashtriya chemical and fertilizers, Bambay was used for the study.

Sample prepareation for ^{15}N assay

The plant samples were digested with appropriate reagents as per Kjeldahl's method to convert organic nitrogen into ammoniacal form (Bremner, 1965). On completion of nitrogen distillation, and absorption of ammonia in 1 per cent boric acid solution, the samples were titrated with 0.02N H_2SO_4 to obtain nitrogen in the form of ammonium sulphate which is suitable for ^{15}N assay in the mass spectrometer (Buresh *et al.* 1982). The acidified boric acid solution containing ^{15}N nitrogen in the form of ammonium sulphate was evaporated at 60°C to dryness, cooled to room temperature and stored in glass vials.

Conversion of ammonia to nitrogen gas

The conversion of ammonium to N^2 gas for mass-spectrophotometric analysis was accomplished by treating the sample in the vial in its powdered state with alkanline sodium hypobromite solution in the complete absence of air. The N_2 gas was evolved as described below.

$$2NH_3 + 3\ NaOBr = 3\ NaBr + 3H_2O + N_2$$

^{15}N analysis

Mass spectrophotometer (Micromass 622 V.G. 150 GAS) was used for the study. The principle, description and micromass 622 was outlined by Buresh *et al.* (1982) and Pruden et al. (1985)

Determination of isotopic composition

The pressure of N_2 gas in the micromass 622 gas inlet for proper level for analysis was accomplished with a variable volume, stainless steel bellow that was evacuated before the entry of N_2 gas. After N_2 entered the bellow, the gas inlet valve was closed and the bellow was adjusted to increase the pressure. The gas was then allowed to enter through a fine stainless steel capillary tube followed by entry into the ion source of the mass spectrometer and was ionized by electron impact. The ions of interest formed were ($^{14}N^{14}N$) with mass 28 and (^{14}N ^{15}N) with mass 29. As these ions have ion source they were directed towards the mass analyzer having high voltage electrostatic field. ^{15}N analysis results were calculated as described by Pruden *et al.* (1985).

Results and Discussion

Two cultures isolated from the flooded rice soil were characterised. The first isolate was straight, terminal spore forming gram positive rods which produced acetate and propionate as UFA and utilized sucrose more efficiently than the other carbon sources. This culture also exhibited nitrogenase activity. Based on the morphology, the culture was identified as *Clostridium.*

Table 1. Amount of N fixed by *Clostridium* and *Klebsiella*, labelled N treatment for a period of 30 days

Treatments	Total N uptake (mg pot***-1)	% N dff*	^{15}N recovered from fertilizer source (mg pot^{-1})	N contributed from soil + atmosphere sources (mg pot^{-1})	N_2 fixed	
					mg pot^{-1}	kg ha^{-1} m^{-1}
Uninoculated (100 kg ^{15}N ha^{-1})	**54.360	24.82	13.492	40.868	-	-
(100 kg ^{15}N ha^{-1}) *Clostridium*	64.636	16.25	10.503	54.133	13.265	4.422
(100 kg ^{15}N ha^{-1}) + *Klebsiella*	62.465	20.97	13.101	49.347	8.506	2.835

* - % Nitrogen derived from fertilizer **- ^{15}N urea at 10 atom % ^{15}N excess

*** - 6 kg soil pot^{-1}

Burns (1982) described *Clostridium pasteurianum* from flooded rice ecosystem and *Clostridium* is considered to be more widely distributed than Azotobacter. Another isolate with glistening and raised colonies in the roll tubes were found to be gram negative straight rods. During growth, they released volatile intermediates like acetate and formate along with CO_2 and H_2. The ARA activity of the culture found to be positive. Based on these characters, they were identified as *Klebsiella* sp. (Marimuthu, 1995).

The results of the pot culture study with immobilized nitrogen fixing facultative and obligate anaerobic *viz. Klebsiella* and *Clostridium* using ^{15}N technique clearly indicated that total N uptake was considerably increased due to inoculation of nitrogen fixing anaerobes. The total N uptake of the *Clostridium* inoculated was higher (64.636 mg pot^{-1}) than *Klebsiella* inoculation (62.475 mg pot^{-1}). The per cent N derived from fertilizer (% N dff) was remarkably low in *Clostridium* (10.503). In *Klebsiella* %N dff was 13.101 which is lower than uninoculated fertilized treatment (13.492). The computed results indicated that the higher N_2 fixation was by *Clostridium* sp. (4.422 kg^{-1} ha^{-1} $month^{-1}$) than by *Klebsiella* sp (2.835 kg^{-1} ha^{-1} $month^{-1}$) over control.

The extent and significance of biological nitrogen fixation by anaerobic bacteria in soil has however, not been established, although many investigators have provided an excellent basis for postulation. In general, significant nitrogen fixation under aerobic and anaerobic conditions occur when soluble carbohydrates are present (Chang and Knowles, 1965). Rice *et al.* (1967) reported that N_2 fixation in soil can occur and the rate equivalent to 13-150 kg ha^{-1} was observed when the soil was amended with 1 per cent straw or less. However, the net N_2 fixation is debated on both sides. The present investigation was carried out to establish the importance of anaerobic nitrogen fixation in the flooded soil and its contribution to the plant. Nitrogen is fixed in the rhizosphere of rice plant and translocated into plant tissues. Fixed nitrogen was found even in the earheads at a fairly short time after the ^{15}N application. Ito *et al.* (1980) reported that ^{15}N incorporation in rice plants under water culture conditions, 10 per cent of the fixed nitrogen was translocated to rice plants. In the present study both the facultative and obligate anaerobes augmented the N uptake. The pot culture experiments showed that *Clostridium* increased the uptake 4.22 kg N ha^{-1} $month^{-1}$ and *Klebsiella* recorded 2.835 kg N ha^{-1} $month^{-1}$. This findings suggested that nitrogen fixed by these nitrogen fixers in the rhizosphere can be utilized by the rice plant rapidly and favour the N_2 supply from the atmosphere.

The prolonged submerged rice system is favourable for reducing the soil nitrogen into ammoniacal form. However, the existence of facultative and obligate anacrobes could also favour the fixation of atmospheric nitrogen into ammoniacal form which could be utilised by the growing rice plants. This fixation is clearly evidenced with the increased soil N contribution. Though the N_2 fixation by the anaerobes seems to be a meagre quantity if the mechanism of fixation is known explicitly, the phenomenon could be improved. In the present investigation, it is observed that 19 to 25 per cent of the N mobilized by the plant is derived from atmosphere by N_2 fixation. Hence more information is needed for capitalising this process fully.

References

Bergersen, F.J. 1980. Methods of evaluating biological nitrogen fixation. (Ed.) F.G.Bergerson. Wiley interscience Publication New York. p.700.

Bremner, J.M. 1965. Organic N fractions. In : Methods of soil analysis.Part II (Ed.) C. A. Black Am. Soc. Agron. Inc. Madison - Wisconsin, USA, pp 11-1255.

Buresh, R.J., Austin, E.R. and Craswall, E.T. 1982. Analytical methods in ^{15}N research. *Fert. Res.* **3**: 37-62.

Burns, R.G. 1982. Enzyme activity in soil and possible role in microbial ecology. *Soil Biol. Biochem.* **14** : 107-108.

Chang, P.C. and Knowles, R. 1965. Non-symbiotic nitrogen fixation in some quebec soil. *Can. J. Microbiol,* **11** : 29-38.

Grist, D.H. 1965. Rice. Longmans, Green Co. Ltd. pp 221-233

Holdemann, L.V., Cata, E.P. and Moore, W.E.C. 1977. Anaerobes Laboratory Manual Vol. IV. VPI Anaerobic laboratory, Vigina Polytechnic Institute and State University, Blacksburg, Virginia.

Hungate, R.S., 1975. Microorganisms in the remen of cattle fed at a constantration. *Can. J. Microbiol.* **3** : 289-311

Ito, O., Cabera, D. and Wantanabe, I. 1980. Fixation of dinitrogen ^{15}N associated with rice plants. *Appl. Environ. Microbiol,* **39**: 554-555

Koyama, T. 1963. Gaseous metabolism in sediments and paddy soil and the production of atmospheric methane and hydrogen. *J. Geophys. Res.* **68** : 3971-3973.

Lee, K.K. and Wantanabe, I. 1977. Problems of acetylene reduction technique applied to water saturated paddy soils. *Appl. Environ. Microbiol,* **34** : 654-660

Mac Rae, I.C. and Castro, T.F. 1967. Root exudates of the rice plant in relation to Akagare, a physiological disorder or rice. *Pl. Soil,* **26** : 317-323.

Marimuthu, P. 1995. Anaerobes in low land flooded rice soil ecosystem. Ph.D. Thesis submitted to TNAU, Coimbatore 3 p. 137

Matsuguchi, T. 1979. Factors effecting heterotrophic nitrogen fixation in submerged rice soils. Nitrogen and Rice. International Rice Research Institute, Los Banos, Philippines, pp. 207-222.

Pruden, G., Powlson, D.S. and Jenkinpon, D.S. 1985. The measurement of N in soil and plant material. *Fert. Res.* **6** : 205-218.

Rice, W.A., Paul, E.A. and Wetter, L.R. 1967. The role of anaerobics is in symbiotic nitrogen fixation. *Can. J. Microbiol,* **13**: 829-836.

Skinner, F.A. 1986. Anaerobic bacteria and their activities in soil. In : Soil Microbiology (Ed.) N. Walker, Buffet worthes, London, p 1-17.

Wada, H., Panichsapatana, S., Kimura, M. and Takai, Y. 1978. Nitrogen fixation in paddy soils. part I. controlling factors affecting N_2 fixation. *Soil. Sci. Pl. Nutr.* **24** : 357-365.

Yashida, T. and Ancajas, R.R. 1971. Nitrogen fixation by bacteria in the root zone of rice. *Soil. Sci. Soc. Am. Proc.* **35** : 156 - 157.

(Received: March 1998; Revised: November 2001)

Madras Agric. J. 88 (10-12) : 621-624 October-December 2001

Isolation and characterization of nitrogen fixing anaerobes and facultative anaerobes from paddy ecosystem

P. MARIMUTHU, M. MAHESWARI, P. DORAISAMY AND S. GUNASEKARAN
Dept. of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore - 641 003.

Abstract : The importance of nitrogen fixing activity of *Clostridium pasteurianum and Klebsiella pneumonia* in flooded soils is known for decades. The anaerobic and facultative anaerobic nitrogen fixing microorganisms were isolated from the flooded paddy ecosystem. Their growth on carbon sources, CO_2 production, denitrification, volatile fatty acid production, nitrogenase activity and protein profile of the cultures were estimated and characterized as *Clostridium and Klebsiella.* (*Key words*: anaerobic, facultative anaerobic, nitrogen fixation, *Clostridium, Klebsiella*).

Some of the soil anaerobic micro organisms fix atmospheric nitrogen, decompose cellulose, form methane or reduce sulphate and nitrate are of special interest in the flooded rice soil (Skinner, 1986). Nitrogen fixing microorganisms enrich nitrogen in submerged rice soils by atmospheric nitrogen fixation (Grist, 1965). Yoshida and Ancajas (1971) reported that in flooded soil in which rice is growing, some nitrogen apparently occurs in the root rhizosphere. Paul and Newton (1960) reported a number of nitrogen fixing soil organisms such as *Pseudomonas,* facultative bacilli and *Klebsiella* sp. Rice *et al.,* (1967) isolated the nitrogen fixing *Clostridia* from rice soil. *Clostridium pasteurianum* and *Klebsiella pneumonia* are of particular interest because of their ability to fix atmospheric nitrogen. An attempt was made to isolate and characterize the nitrogen fixing anaerobes and facultative anaerobes from the paddy ecosystem.

Materials and Methods

Isolation of nitrogen fixing anaerobes and facultative anaerobes from paddy ecosystem.

Soil samples at random were collected from the rice during active tillering stage at lower horizon (10 cm depth) under anaerobic conditions (Ramasamy *et al.* 1992) for the isolation of anaerobic nitrogen fixing microflora. Nitrogen fixing anaerobes were isolated using Hungate's (1957) roll tube technique using Hill's medium (Bergerson, 1980). The individual colonies developed in roll tubes were transferred to the vials containing nitrogen free medium and inoculated under nitrogen atmosphere. After the desired growth, the cultures were further purified using roll tube technique.